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OIL SHALE INDUSTRY WASTEWATER: IMPACT ON RIVER MICROBIAL COMMUNITY AND POSSIBILITIES FOR BIOREMEDIATION

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in Microbiology) on May 18th, 2004 by the council of the Institute of Molecular and Cell Biology, University of Tartu.

Opponent: Dr. Alexander Boronin (Institute of Biochemistry and Physiology of Microorganisms of Russian Academy of Sciences)

Commencement: June 29, 2004

Publication of this dissertation is granted by the University of Tartu

Tartu Ülikooli Kirjastus
www.tyk.ut.ee
Tellimus nr. 265

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers that will be referred by their Roman numerals in the text.

I Truu J., Alamäe T., Heinaru E., Talpsep E., Kokassaar U., Tenno T., Heinaru A. (1997) Impact of oil shale mine water on microbiological and chemical composition of north-eastern Estonian rivers. Oil Shale, 14, 4, 526–532.

II Truu J., Heinaru E, Talpsep E, Heinaru A. (2002) Analysis of river pollution data from low-flow period by means of multivariate techniques: a case study from the oil-shale industry region, norteastern Estonia. Environmental Science and Pollution Research, 1, 8–14.

III Truu J., Talpsep E., Heinaru E., Heinaru A. (2002) Self-purification processes in Estonian rivers receiving sewage from oil shale processing industry. Large Rivers, 13, 3–4, Archiv für Hydrobiologie Suppl. 141/3–4, 459–469.

IV Truu J., Talpsep E., Heinaru E., Stottmeister U., Wand H., Heinaru A. (1999) Comparison of API 20NE and Biolog GN identification systems assessed by techniques of multivariate analyses. Journal of Microbiological Methods, 36, 3, 193–201.

V Truu, J**.,** Kärme, L., Talpsep, E., Heinaru, E., Vedler, E., Heinaru, A. (2003) Phytoremediation of solid oil shale waste from the chemical industry. Acta Biotechnologica, 23, 2–3, 301–307.

VI Truu J., Talpsep E, Vedler E., Heinaru E., Heinaru A. (2003) Enhanced biodegradation of oil shale chemical industry solid wastes by phytoremediation and bioaugmentation. Oil Shale, 20, 3, 421–428.

ABBREVIATIONS

BOD	biological oxygen demand
COD	chemical oxygen demand
CIA	co-inertia analysis
PAH	polycyclic aromatic hydrocarbon
PCIA	Procrustean co-inertia analysis
PC	principal component
RAPD	randomly amplified polymorphic DNA
S	recorded number of species
S*	expected number of species

1. INTRODUCTION

Human activities affecting the quality and quantity of water in rivers and streams are wide and diverse. The input of unpurified or partly purified wastewater from human settlements and industry, and pollution from transportation and agriculture results in enrichment in water bodies of nutrients and organic and inorganic compounds. In Estonia, the decline of industrial and agricultural production, as well as the reconstruction of wastewater treatment systems during the last decade has decreased the pollution load to rivers. Because the main waste generators in Estonia are oil shale mining, and the oil shale chemical and energy industries, oil shale ashes and semi-coke dominate hazardous wastes.

More than 70 years of oil shale thermal processing has resulted in huge dump sites of the coke ash (semi-coke) from semi-coking of oil shale in the areas surrounding oil shale chemical industry plants in northeastern part of Estonia. The semi-coke mounds cover an area about 200 ha and contain up to 100 million tons of solid waste. Currently, about 600 000 tons of processed semi-coke is disposed annually. Semi-coke solid wastes contain several organic and inorganic compounds (oil products, asphaltenes, phenols, PAHs, sulfuric compounds). Although the production of oil shale energy and oil has decreased steadily during the last years with a corresponding decrease in wastes (12.7 million tons per year in 1995 to 8.1 million tons in 2002, Statistical Office of Estonia, 2003), the semi-coke mounds constitute one of the major adverse environmental challenges in Estonia (Estonian Environment Information Centre, 2001). The leachate from these mounds, formed from flushing water and precipitation, contains variety of organic and inorganic compounds (oil fractions, phenols, sulphides, PAHs etc.). Presently the phenol-rich leachate (from 300 up to 10 000 m^3 day^{-1}) from the drainage system of semi-coke mounds is discharged via channels and the Kohtla and Purtse rivers into the Baltic Sea without treatment. This wastewater has been affecting the Purtse River watershed for decades. An estimated 12 tons of phenols enters the Gulf of Finland annually from semi-coke mounds via the Kohtla and Purtse rivers (Kundel and Liblik, 2000). The bottom sediments in these rivers contain elevated total PAH, ranging from 4,270 to nearly 150,000 μg kg^{-1} dry sediment (Huuskonen *et al.*, 2000). The pollution from depository deteriorates not only the streams, but also the underlying aquifers.

Another source of pollution to rivers in this area is mine water (ca 2×10^8 m^3 $year^{-1}$), which increases water mineralization and sulphate concentration (Erg, 2003) as well as heavy metal concentration in sediments (Szava-Kovats, 2001, 2002). Nearly 100 tons of sulfates and 50 tons of oil products are discharged with mine water to local water bodies annually (Narusk and Nittim, 2002).

The reserves of oil shale in the world are estimated in the amount of 10^{13} tons, i.e. they exceed the resources of other solid fuels (coal, lignite, brown

coal) all taken together (Veiderma, 2003). Countries like Australia, Jordan, China and United States are considering to exploit their oil shale deposits for production of oil (Bsieso, 2003; Schmidt, 2003; Qian et al., 2003). The creation of a new energy industry in countries rich in oil shale resources arise environmental issues and therefore the knowledge obtained in Estonia with respect to oil shale industry impact on natural resources has great practical value for sustainable development of this industry in other countries.

The general objective of this thesis was to assess the impact of oil shale industry wastewater on river microbial community structure and functioning in northeastern Estonia.

The specific aims were:

1. To evaluate the impact of oil shale underground mine water on microbiological characteristics of river lotic bacterial community.
2. To evaluate the degradation of phenols from oil shale processing industry in river system and describe the response of the river bacterial community to pollution load from semi-coke dump area.
3. To examine the abundance of biodegradative bacteria, their diversity and distribution within the river system along the pollution gradient.

2. LITERATURE REVIEW

2.1. River microbial community

In contrast to our knowledge of marine and fresh water lake microbiology, the ecological impact of planktonic and attached bacteria within river ecosystems is poorly understood and less extensively studied (Left, 1994). Running waters are subject to quick physicochemical changes due to water fluxes and to the exchanges with surrounding ecosystems. These fluctuations in physicochemical parameters generate changing local spatial and temporal structures and make necessary a continuous adaptation for the river microbes. Among the aquatic microbial communities, the number of studies of river bacteria has been increasing during the last years, especially in relation to environmental pollution (Foreman *et al.*, 1998; Lopez-Archilla and Amis, 1999, Peel and Wyndham, 1997; Pennington *et al.*, 2001). The majority of studies of lotic bacteria have utilized assemblage-level approach (biomass, production, activity), while taxonomic diversity of bacterial community have been more thoroughly studied during last years. Limited understanding of river bacterial community diversity resulted from methodological limitations until the introduction of molecular methods into analysis of microbial diversity in environmental samples in 1990-ties. Molecular approaches, mostly based on 16S ribosomal DNA sequence analysis allow direct investigation of microbial community structure and diversity, as well as quantify the individual species of microorganisms (Theron and Cloete, 2000; Dahllöf, 2002).

Stream ecosystem is spatially heterogeneous and consists of several subsystems, that include the central surface stream, and vertically and laterally arrayed saturated sediments (hyporheic and parafluvial zones) (Fisher *et al.*, 1998). According to these subsystems stream/river microbial community could be divided roughly into three compounds. Firstly, lotic microbial community, which could in turn divided into free-living and attached microorgansims. Secondly, depending on river morphometry the sediment microbial community may play significant role in nutrient cycles. And the third compound is biofilm, like epilithion — the biofilm which covers stones in aqueous environment. As stream subsystems are hydrologically connected and water moves through all of these subsystems as it flows downstream, also microbial communities of different subsystems are connected and exposed to dissolved and suspended load.

Earliest works on characterization on river lotic bacterial community species composition were carried out by group leaded by Prof. Maxine Holder-Franklin (Holder-Franklin *et al.*, 1978, 1981). She used numerical taxonomy for grouping of aerobic heterotrophic bacteria isolated by cultivation from river water. Most recent studies indicate that rivers have a specific planktonic bacterial community distinct from bacteria in neighboring environments like soil or sea water as shown by analysis available database of 16S rDNA sequences from freshwater bacterioplankton (Zwart *et al.*, 2002). Typically

freshwater habitats harbor bacterial species from the *Proteobacteria,* the *Cytophaga-Flavobacterium-Bacteroides* group, the *Actinobacteria* and the *Verrucomicrobia* divisions. The number of different dominant bacterial species in riverine bacterioplankton is around 50 according to PCR-DGGE analysis (Dumestre *et al.*, 2001, Troussellier *et al.*, 2002). Most recent studies have shown that river bacterial community is dominated by *β-Proteobacteria* and *Cytophaga-Flavobacterium* cluster both in sediments and water column (Araya *et al.,* 2003; Brümmer *et al.,* 2000, 2003; Sekiguchi *et al.*, 2002). Hyporheic zone of streams and rivers is dominated by *α-Proteobacteria*, and second by abundance are *γ*- and *β-Proteobacteria* (Feris *et al.*, 2003). River biofilm communities are dominated by members of *α*-and *β-Proteobacteria*, and *Cytophaga* group (Battin *et al.*, 2001; Manz *et al.*, 1999). Most abundant bacterial group in river lotic organic aggregates ('river snow') is constituted by *β-Proteobacteria*, followed by importance by *Cytophaga-Flavobacteria* group (Bockelmann *et al.*, 2000).

Compared to bacteria, archaeal community in rivers has been studied in few cases. The Archaeal species found in rivers are related to freshwater species inhabiting lake plankton and sediments, and belong mainly to the kingdom Crenarchaeota (Crump and Baross, 2000; Abreu *et al.*, 2001).

If to compare activity of free-living, sediment and biofilm bacterial communities in streams, then biofilm and sediment populations tend to be more abundant and active (Bell *et al.*, 1981; Haglund *et al.*, 2002). Attached and particle-associated bacteria are 100 times more active than free-living bacteria and can account up to 90% of heterotrophic bacterial activity (Crump *et al.,* 1999; Crump and Baross 2000). Biofilm growth increases hydrodynamic transient storage (streamwater detained in quiescent zones) and the retention of suspended particles as well as enhances the relative uptake of organic molecules of lower bioavailability (Battin *et al.,* 2003).

Diversity and abundance of bacterial community in rivers (streams) may vary with respect to season (Bell *et al.,* 1980, 1982). Highest cell numbers and biomass occurs in early summer (~10^{10} cells L^{-1}, Kirschner and Velimirov, 1997) or annual trend may be bimodal (Leff *et al.,* 1998). Holder-Franklin and Franklin (1993) studied river bacteria temporal dynamics using time series analysis and found that seasonal component accounted for 40 to 55% of total variance for bacterial plate counts. In addition they discovered periodic fluctuations in bacterial numbers with period 6–7 weeks, which could not be linked to water physico-chemical parameters. Seasonal changes vary between different rivers due to differences in catchment's area, discharge or local land-use patterns. Seasonal dynamics of riverine bacterioplankton may be related to allochthonous input or input from the sediments. In case of biofilm the temperature-dependent parameters are considered triggering the temporal community shifts (Brümmer *et al.,* 2003). The temporal dynamics on species level is generally dissimilar to assemblage level variation. Liu and Lemke (2002) studied temporal changes in the abundances of *Pseudomonas putida* and

Acinetobacter calcoaceticus and found different seasonal patterns both between studied two species as well as between species' and assemblage level parameters.

Besides seasonal factors, bacterial community structure and activity in rivers and streams is related to organic carbon and inorganic nutrients' concentrations (bottom-up control), but the response of bacterial community to the changes of these factors depends on river type (Bell *et al.*, 1982; Castillo *et al.*, 2003; Mohamed and Robarts, 2003). Dominant process of mortality for autochthonous river bacteria is grazing by protozooplankton (top-down control) (Menon *et al.*, 2003).

2.2. Biodegradation in rivers

Contaminants entering rivers may break down or become diluted due to self-purification by physical, chemical, and above all, biological processes. The capacity to degrade a great variety of recalcitrant compounds is particularly harboured in bacteria (Romantschuk *et al.*, 2000; Watanabe, 2001). Biodegradation in river water depends — in addition the type and structure of the chemical compounds — on the available micro-organisms and the environmental conditions (Holder-Franklin, 1992).

From environmental factors temperature, water pH, concentration of electron acceptors and mineral nutrients influence the rate of biodegradation (Yuan *et al.,* 2004). For example, rate of biodegradation of nonylphenol polyethoxylate in river water increased from 68% at 7°C to 96% at 25°C (Manzano *et al.*, 1999), also biodegradation kinetics of linear alkylbenzene sulfonates is temperature-dependent (Perales *et al.,* 1999). Availability of mineral nutrients, typically nitrogen, phosphorous and sulphur increases rate of biodegradation in river water (Žgajnar and Zagorc-Končan, 1999; Yuan *et al.,* 2001).

Generally the degradation kinetics of xenobiotics in river water and sediments is best explained by first order model, but it may not be applicable for the compounds with a poor biodegradability (Börnick *et al.*, 2001, Toräng *et al.*, 2002). The second order model describing the dependence of the biodegradation rate of xenobiotics on both its concentration and its bacterial biomass is based on the kinetics of the first-order biodegradation reaction and according to some authors does not apply in river case (Četkauskaite *et al.*, 1998). However, there are indications that in case of some xenobiotics like bisphenol A the biodegradation is influenced by bacterial biomass (Kang and Kondo, 2002). While modeling/estimating the biodegradation in river the biofilm activity should be considered and when necessary, incorporated into the model. The share of biofilm to the removal of xenobiotics is dependent on river morphology as well as on type of xenobiotic (Boije *et al.*, 2000).

Usually only the chemical determinations are made for assessment of pollutant attenuation, while functional status of the degradative microbial community remains unknown. In order to assess the biological detoxification of pollutants, knowledge about the impact of pollutant loading upon the autochthonous microbial communities as well as upon key micro-organisms within the remediating consortia, is needed (Whitley and Bailey, 2000). Microbial community function in regard of pollutants degradation depends in addition to community taxonomic composition also in genetic diversity of functional/ catabolic genes in environment (Junca *et al.*, 2003; Watanabe *et al.*, 2002). Study of catabolic genes diversity (variability in substrate specificity, inducer specificity, number of catabolic routes, kinetics of catabolic enzymes) is needed for understanding of microbial functioning and degradation processes in the environment (Widada *et al.*, 2002). Typically overall microbial diversity is reduced when environment becomes contaminated, while abundance of members of autochthonous microbial community capable of pollutant degradation increase (Picado *et al.*, 2001, Ruberto *et al.*, 2003; Saagua *et al.*, 2002). The diversity of pollutant degraders could be assessed with culture based and molecular methods that complement each other and allow for better understanding of biodegradation in the environment (Watanabe and Hamamura, 2003).

2.3. Data analysis in river microbiological studies

While studying diversity of microbial communities in rivers and streams, the complex data sets build up. These data sets become more and more sophisticated with development of molecular techniques in microbial ecology. In river microbiology multivariate techniques were introduced by Prof. Maxine Holder-Franklin (Holder-Franklin, 1985; Holder-Franklin and Wuest, 1983). She used in her research factor analysis followed by path analysis or regression analysis for describing bacterial populations shifts. Applying mathematical methods she was able to link environmental parameters with river microbiological data. The weakness of such approach is that in analysis first stage environmental variables are processed irrespective of microbiological variables and this may lead to underestimation of impact of some measured environmental parameters on microbial community dynamics. This problem could be solved by applying canonical analysis methods ("direct gradient analysis" methods or "constrained ordination" methods), like canonical correlation analysis, redundancy analysis, and canonical correspondence analysis, but these methods became available with the spread of personal computers. In canonical analysis biological and environmental data tables are analyzed simultaneously, and it allows relating community species composition to environmental factors. The choice of the particulate method for data analysis depends on the character of data sets. For example, if data table contains many zeros it should be processed using

correspondence analysis instead of Principal Component Analysis (PCA) or appropriate data transformation should be applied prior PCA (Legendre and Gallagher, 2001).

One of the key issues in case of multivariate methods is the rank of the data matrices under investigations. If the number of observations (samples) is smaller than descriptors then the matrix is not of full rank and data analysis should be performed according to this situation. Such types of data matrices are typical in environmental microbiology, like water samples with bacterial species (sample by species table) or tables with species characteristics obtained with different methods (strain by characteristic table). Interpretation of such data sets needs hypothesis testing methods that are based on randomization, permutation or bootstrapping (Monte Carlo methods) (Manly, 1997). When the aim of the researcher is to take into account information simultaneously from two or more data tables which are not of full rank, the data analysis methods under consideration are Mantel test, co-inertia analysis and Procrustean superimposition approach. The Mantel test provides means to test the association between distance matrices and has been widely used in ecological and evolutionary studies. The method is particularly suitable when one or both data sets are in form of distance matrices. Procrustean superimposition and co-inertia analysis has an additional feature allowing visualizing the analysis results and examining the concordance of observations (samples) (Peres-Neto and Jackson, 2001; Dray *et al.*, 2003a). Also the importance of each variable (characteristic) on outcome of data analysis could be evaluated. Applicability of these methods is limited as they are not readily available in standard statistical packages and researchers tend to neglect the characteristics of collected data sets and the objectives of the data analysis (Dray *et al.*, 2003b).

3. THE STUDY AREA

In the first study, conducted in 1988–1991, river water samples were collected from four locations (Fig. 1A). The second study was carried out on the Purtse River system during the period from May to September 1995. Water samples were collected six times from ten sampling sites (Fig. 1B).

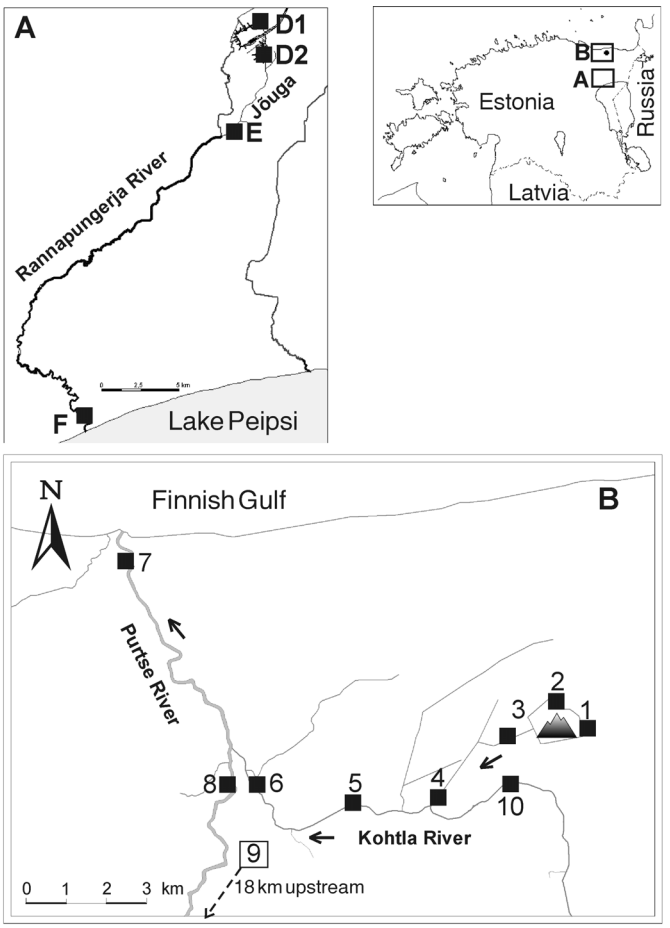

Figure 1. The maps of study areas. A. The Rannapungerja and Jõuga rivers and sampling sites. Legend: D1 – reservoir inflow; D2 – reservoir outflow; E – Jõuga River; F – Rannapungerja River. B. The Purtse River catchment area and sampling sites. Legend: 1 – plant area; 2 – channel beginning; 3 – channel middle; 4 – channel end; 5 – Kohtla River (after joining with the channel); 6 – Kohtla River (end), 7 – Purtse River (after joining the River Kohtla); 8 – Purtse River control I; 9 – Purtse River control II; 10 – Kohtla River control.

4. RESULTS AND DISCUSSION

4.1. Mine water impact on river water microbial community (Publication I)

In order to get better insight how mine water affects water bodies in north-eastern Estonia data set analysed in Paper **I** were appended with data from sedimentation reservoir inflow (water pumped out from oil shale underground mine) and outflow (ditch directing water to the Jõuga River) (Fig. 1A).

The mean water chemical and microbiological parameters are given in Table 1. Data analysis using Principal Component Analysis (PCA) followed by multivariate randomization test indicated that there are statistically significant ($P<0.001$) differences in water chemical parameters between sampling locations. According to PCA plot (Fig. 2A), the sedimentation reservoir as well as the Jõuga River are characterized by elevated concentration of sulfate, carbonate and calcium, and by higher water hardness compared to the Rannapungerja River. PCA plot of sample scores indicates (Fig. 2B) that major changes in concentration water chemical parameters within studied flow path occur between the Jõuga and Rannapungerja rivers.

Based on data from Table 1 the performance of sedimentation reservoir for mine water treatment was estimated. Removal efficiency was highest for COD, nitrates and iron (45.4–50.9%), followed by BOD (35.1%). Retention of sulfates was 19.8% and reduction of carbonates and calcium was low (2–5%).

Tabel 1. Average values and standard deviations of water microbiological, chemical and physical parameters from two studied rivers and sedimentation reservoir.

Variable	Site			
	Reservoir inflow	Reservoir outflow	Jõuga River	Rannapungerja River
Total count (10^6 cells ml^{-1})	5.1±2.5	4.7±1.7	4.1±1.8	4.5±3.2
Viable heterotrophs (10^3 cfu ml^{-1})	20.6±14.2	18.6±15.7	8.1±6.7	8.9±11.5
Denitrifying bacteria (10^3cells ml^{-1})	1.6±1.4	12.4±29.2	1.04±1.01	0.54±0.97
Lipolytic bacteria (10^3 cells ml^{-1})	4.0±4.7	16.1±28.7	4.2±6.3	4.3±8.9
Cellulolytic bacteria (cells ml^{-1})	28±26	140±150	340±580	510±790
Coliform bacteria (10^3 cells l^{-1})	1.5±1.2	2.0±1.00	1.4±0.9	0.9±0.6
Oxygen (mg l^{-1})	12.4±1.3	12.6±0.8	11.3±0.7	10.8±2.0
pH	7.9±0.3	7.9±0.2	7.8±0.6	7.6±0.5
BOD_5 (mgO_2 l^{-1})	1.85±0.38	1.22±0.47	1.48±0.67	2.36±0.97
COD_{Mn} (mgO_2 l^{-1})	5.9±3.6	3.1±0.6	8.6±3.4	22.1±10.1
NO_3^- (mg l^{-1})	3.85±2.50	2.1±2.60	1.23±1.27	1.16±1.08
Cl^- (mg l^{-1})	27.8±5.5	29.5±5.5	28.4±8.9	15.5±10.7
SO_4^{2-} (mg l^{-1})	333±77	267±19	207±108	63±44
HCO_3^- (mg l^{-1})	6.7±0.4	6.4±0.2	6.0±0.8	3.7±1.4
Ca^{2+} (mg l^{-1})	5.8±1.1	5.7±1.1	6.1±1.9	3.6±1.3
Fe^{3+} (mg l^{-1})	0.55±0.58	0.27±0.14	0.33±0.09	0.71±0.33
Load (m^3 s^{-1})	0.34±0.07	0.34±0.07	1.80±0.68	6.00±6.67

Water microbiological data set was analysed in same way as chemistry data. According to multivariate randomization test the studied locations differ in microbiological parameters ($P<0.001$). Water from sedimentation reservoir is characterized by higher numbers of aerobic heterotrophic bacteria and denitrifying bacteria as well as by more abundant lipolytic bacteria (Fig. 2C). Major source of variation in microbiological data set is related to differences between sedimentation reservoir inflow and outflow and river locations (Fig. 2D). This suggests that despite the large amount mine water discharged to the Jõuga River, the river microbial community is not strongly altered.

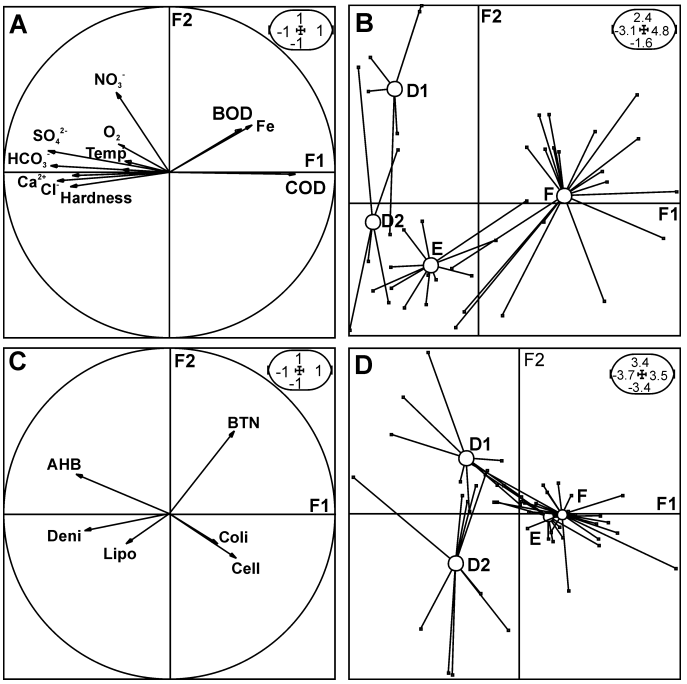

Figure 2. Ordination of the sampling sites based on PCA of measured water chemical and microbiological variables. The results of PCA are interpreted using scatter of the samples scores connected with the site centroids (star plot), and via correlation circles. **A.** Correlation of the water chemical and physical variables with two first axes (F1 x F2) of the PCA. The first and second PCs describe 87.0% and 10.8% of the overall data variation respectively. **B.** Sampling sites along F1 x F2 plane. Each sampling site is represented by the centroid of the sample scores for given site over a study period. **C.** Correlation of the water microbiological variables with two first axes (F1 x F2) of the PCA. The first and second PCs describe 37.2% and 20.2% of the overall data variation respectively. **D.** Sampling sites along F1 x F2 plane. Abbreviations: AHB – aerobic heterotrophic bacteria, BTN – bacterial total number, Deni – denitrifying bacteria, Cell – cellulotic bacteria, Coli – coliform bacteria, Lipo – lipolytic bacteria.

The effect of mine water on river microbial community was clearly visible during underground mine burning. In both rivers maximum numbers of heterotrophic aerobic bacteria ($30x10^3$–$63x10^3$ cells ml^{-1}) and lipolytic bacteria ($95x10^3$–$250x10^3$ cells ml^{-1}) were recorded just after the beginning of underground oil shale burning.

In Paper **I** the time course of microbiological parameters was assessed based on dynamics of principal component (PC) scores. First two principal components accounted for 53 % of total variance in microbiological data set. First PC is related negatively to the number of denitrifying bacteria, lipolytic bacteria and viable heterotrophs and positively to the number of cellulolytic bacteria. Second PC is characterized by positive loadings of the counts of total bacteria and coliform bacteria.

In both rivers scores of the first PC had low values in autumn of 1988 (Fig. 3) which coincides with accidental underground oil shale burning. This suggests that the first PC reflects the effect of oil shale mine water on the river lotic microbial community. The second PC can be identified as domestic wastewater input or runoff from surrounding land. Dynamics of the first PC scores exhibits clear seasonal dynamics. The score values are high during flood period in early spring and decrease towards summer months.

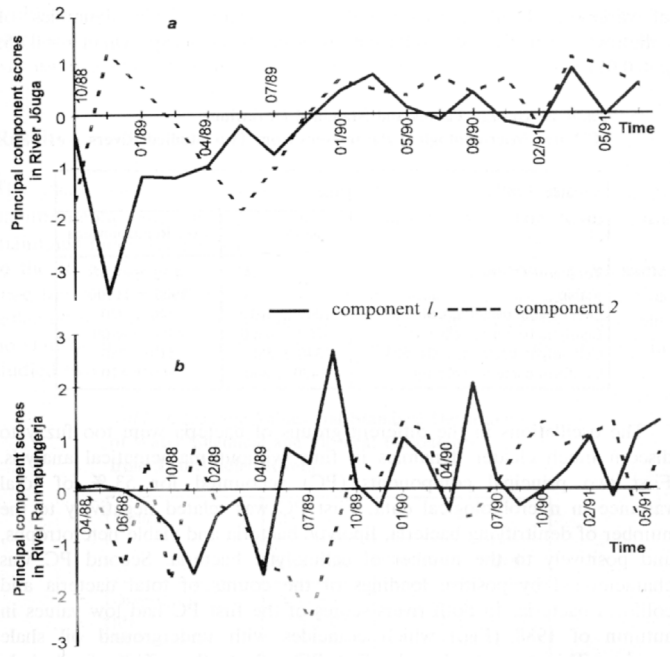

Figure 3. (Paper I, Fig. 1). Dynamics of first two principal component scores in the Jõuga River (a) and in the Rannapungerja River (b).

In order to relate water microbiological data to water chemistry data, co-inertia analysis was performed using only river data set. This analysis indicated statistically significant ($P<0.01$, 10 000 permutations) co-structure between water microbiological and chemical variables. Besides seasonal factors like temperature and load, the microbiological variables were related positively to COD and ammonium concentration. The studied microbiological variables were related negatively to concentration of calcium and bicarbonate in river water.

Main approach to reduce the environmental impact of mine water is to treat this water before the release into environment. Various methods could be used applying microbial processes in natural or engineered systems to achieve effective treatment of mine water. In case of Estonia, considering the large amount of pumped out mine water most reasonable could be the use of passive treatment systems. Passive treatment systems are usually based on wetland ecosystems, either aerobic or anaerobic, and compost bioreactors (Johnson and Hallberg, 2002). These systems have been used mainly for treatment of acid mine drainage. However there are also examples when constructed wetlands are ineffective for reduction of sulfate concentration in mine water and retention of this parameter may be higher in streams receiving water from treatment wetland (36% versus 23%; Mitsch and Wire, 1998).

The main factors affecting remediation of mine water in passive treatment systems are: the presence of organic rich sediment with the proper microbial community, the presence of anaerobic conditions necessary for sulfate-reducing bacteria, and proper mix of carbon sources for short- and long term microbial growth. In addition to these factors the removal rate of sulfate depends on the type mine water and the physical conditions of the system and retention time (Kalin 2001). The drawback of most passive treatment systems is that they offer economically viable treatment in a post-mining landscapes where water load is several magnitudes lower than in case of Estonian oil shale mines.

Data analysis presented in current thesis and Paper **I** suggests that river water chemical and microbiological parameters are influenced by mine water input. In order to ascertain which components of river microbial community are most strongly influenced by mine water input and what are consequences of such disturbance, the additional research is needed applying molecular microbiology methods for the analysis of river water and sediment. Such research should also be performed in sedimentation reservoirs. The results of these investigations could be valuable base for evaluating purification potential of studied water bodies as well as for designing more effective mine water treatment systems

4.2. Impact of wastewater from semi-coke dump area and oil shale chemical industry on river microbial community and biodegradation of pollutants (Publications II and III)

4.2.1. Environmental variables

The chemical variables measured along river system (Fig. 1B) are summarised in Table 1 in Paper **II**. Water pH was fairly uniform in river water, but showed mean values >9 in the channel area. The concentration of dissolved oxygen varied between 0.1 $mgO_2\ l^{-1}$ and 12.5 $mgO_2\ l^{-1}$ with the lowest values recorded near the semi-coke mound. The concentration of dissolved oxygen in the water was very low (less than 1 mg l^{-1}or 5%) in the upper part of the channel, but increased remarkably downstream (mean percent saturation values at sites No. 6 and 7 were 60.1% and 99.8%, respectively). In contrast, the COD was high in the channel and decreased downstream. The concentration of total nitrogen and ammonium nitrogen compounds in the river system varied by more than two orders of magnitude. Both total phosphorous and nitrogen decreased along the channel, although the total phosphorous reached the 'natural level' in the mid-region of the channel, whereas total nitrogen achieved the 'natural level' at the channel's end. Ammonia-nitrogen compounds showed a pattern similar to total nitrogen, being thousands of times higher at the mound area compared with the mouth of the Purtse River. The variation in total phosphorus was also appreciable. During the study period, an average of 62.2 kg of volatile phenolic compounds entered the river daily from the semi-coke depository, while the maximum discharge exceeded 350 kg day^{-1}. The content of both measured phenols was extremely high in the mound area and channel, whereas no phenols were detected at the control sites.

The first two principal components describe most of the overall data variation (92.5%) in the data set of chemical parameters. The first principal component accounts for 81.1% of the total variation and is negatively correlated with the group of variables that exhibited their highest values in the channel area. This component contrasts the most polluted sampling sites (sites No. 1–4) and the more pristine sites (sites No. 5–10) (Fig. 4). The differences between sampling locations were statistically significant ($p<0.05$), whereas the effect of sampling time was insignificant. The values of volatile and total phenol concentrations were regressed on the first two principal components. Both parameters correlate strongly with the first PC ($r=-0.98$, $p<0.05$).

The first records regarding the environmental situation in Purtse River stem from 1967. At this time the wastewater discharged monthly into the river exceeded the current annual discharge, and river water contained 20–30 mg l^{-1} oil and 30–40 mg l^{-1} total phenols (Kamenev *et al.,* 1995). The chemical composition of wastewater from semi-coke heaps is not well defined and

depends on climatic variables and chemical industry process waters used to compact the coking wastes. Earlier studies have shown that the pH of wastewater from the semi-coke depository ranges from 10.2 to 12.3, with a COD value of 2500–3600 mg O_2 l^{-1}, and concentration of total phenols of 500–700 mg l^{-1} (Orupõld *et al.,* 1997), while the phenolic compounds form approximately 9–10% of the COD of the leachate.

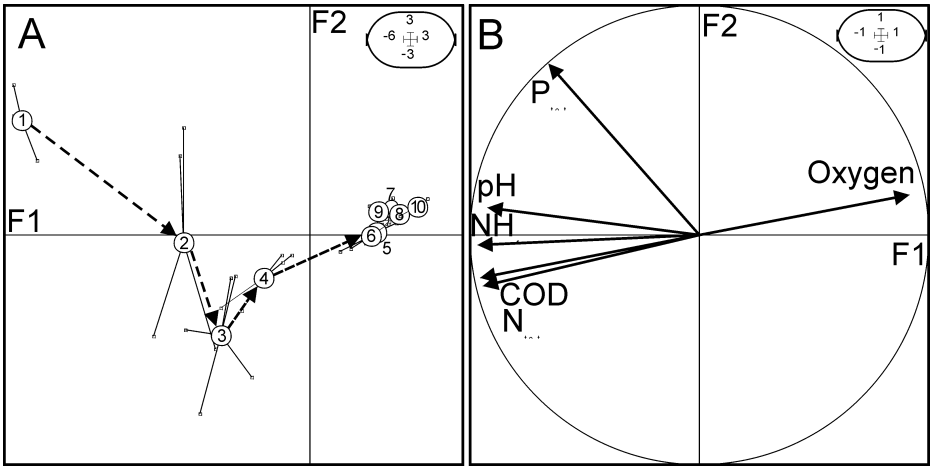

Figure 4. (Paper II, Fig. 2). Ordination of the sampling sites based on Principal Component Analysis (PCA) of chemical data. The results of PCA are interpreted using scatter of the sample scores associated with site centroids (star plot), and via correlation circles. A – Sampling sites along F1xF2 plane. Each sampling site is represented by the centroid of the sample scores for given site over a study period. Dashed lines indicate the flow direction between sampling sites. B – Correlation of chemical parameters with two first axes (F1×F2) of the PCA. The first and second principal components describe 81.1% and 11.4% of the overall data variation, respectively.

Our data are within the same range, except for COD, which showed a wider range (2200–4400 mg O_2 l^{-1}). In addition to elevated pH, COD, phenols, total nitrogen and phosphorous, the wastewater is characterised according to Kahru and co-workers (1997) by high concentrations of sulphuric compounds up to 1300 mg l^{-1}, mainly in the form of sulphates. Recent studies indicate that the contamination level in the Purtse River has not diminished since 1995; maximum concentrations of mono and dibasic phenols reached 0.075 and 0.018 mg l^{-1} in the year 2000, respectively (Estonian Environmental Monitoring Programme, http://seiremonitor.ee).

Principal component analysis indicates that wastewater from semi-coke depository causes increased levels of ammonium, total nitrogen, and COD, as well as a high pH in the channel systems. Volatile and total phenols exhibit a

spatial distribution pattern similar to these four variables, as shown by the strong relationship between the concentration of phenols and the first principal component. Where the channel joins the Kohtla River, these components are diluted 33-fold on average. During the period from 29 May to 02 June 1995, the removal of volatile phenols approached 100% in river water. The decrease in the amount of phenols in water was rapid along the first couple of kilometres in the beginning of the channel area, when roughly half the volatile phenols were removed (Fig. 5). An approximately two-fold reduction in the degradation rate of volatile phenols occurred after the channel joins the Kohtla River. The calculations for the entire study period (May-September 1995) based on data from Kiviter, Ltd. yielded a removal efficiency of 85% for volatile phenols, and 30% for total phenols.

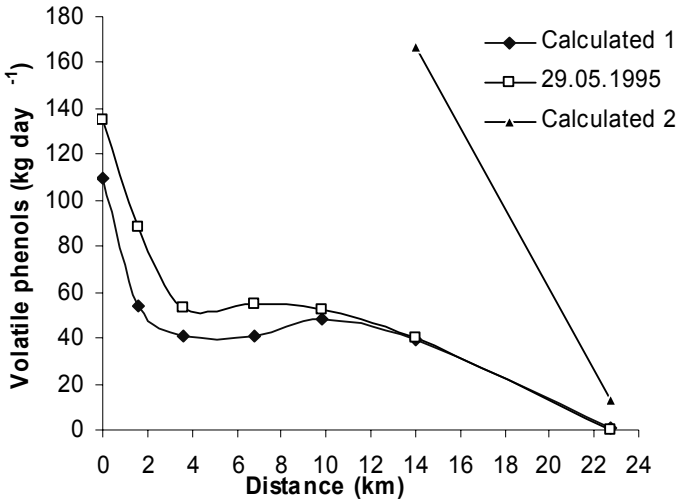

Figure 5 (Paper III, Fig. 2)**.** The dynamics of volatile phenols along river system. Shown are changes in the amount of volatile phenols in 29.05.1995, for the period 29.05–02.06.1995 (line Calculated 1), and for the period 01.05–06.09.1995 (line Calculated 2). The calculations for the second period are based on data supplied by Kiviter, Ltd.

According to the data in the literature, the volatile phenols in the semi-coke leachate mainly contain phenol (from 33.2 to 44.2%) and cresols (from 16.1 to 40.6%), while the concentrations of resorcinol, methylresorcinols and dimethylphenols do not exceed 22.4% (Heinaru *et al.,* 1997). Under aerobic conditions, all phenols (phenol, o, m, and p-cresol, 2,3, 2,4, 2,5, 2,6, 3,4, and 3,5-xylenol) have been observed to degrade in different substrates (Arvin *et al.,*,

1991; Nielsen *et al.,* 1995; Orupõld *et al.,* 2001). Biodegradation of these compounds requires the presence of an active and abundant bacterial community.

Besides biodegradation phenols may be eliminated from river water by various processes including volatilization, adsorption, sedimentation, and photooxidation. But these processes can not account for two time difference in phenol reduction rate within studied river system. One possible explanation could be that in streams of channel area the share of biodegradation by biofilm and sediment bacteria is higher than in Kohtla and Purtse rivers. Small streams have high surface area to volume ratio, and thus biodegradation by biofilms can be much more important than in larger rivers. Streams in channel area possess thick sediment layer (1–2m), which is inhabited even in the beginning of channel where plate counts from water could be obtained occasionally by aerobic heterotrophic bacteria (10^4 CFU ml^{-1}) and phenol degraders ($2x10^4$ CFU ml^{-1}) (Kärme *et al.,* 2003). In deeper sediment layers the phenol degradation may be coupled with sulfate reduction (Vargas *et al.*, 2000, Lin and Lee, 2001).

4.2.2. Microbiological parameters

Variations in the microbiological parameters were greatest along polluted river sections (sites No. 1–6). Direct counts ranged between $4.2x10^4$ and $2.5x10^7$ cells ml^{-1} (Fig. 6). The maximum abundance of bacterioplankton was found at site No. 6. The spatial distribution of aerobic heterotrophic bacteria and phenol degraders is quite similar. In the channel area, the heterotrophic bacterial counts and the number of phenol degraders were very low (<30 cells ml^{-1}), whereas site No. 6 contained the highest values (geometric mean of heterotrophic bacteria and phenol degraders was $1.54x10^5$ and $3.62x10^3$ cells ml^{-1}, respectively). The other biodegradative bacterial groups exhibited a spatial distribution pattern similar to phenol degraders. Colony forming units of biodegradative groups were rarely detectable and their counts did not exceed a magnitude of 10 cells ml^{-1} in the channel around semi-coke heaps during the entire sampling period. A rapid increase in naphthalene, camphor and heptane-degrading bacteria was found at the end of the channel. The biodegradative bacterial population decreased in the Purtse River and was comparable to the bacterial abundance of unpolluted waters in the river system. At the control sites, the maximum geometric means of biodegraders' groups were around 100 cells ml^{-1}.

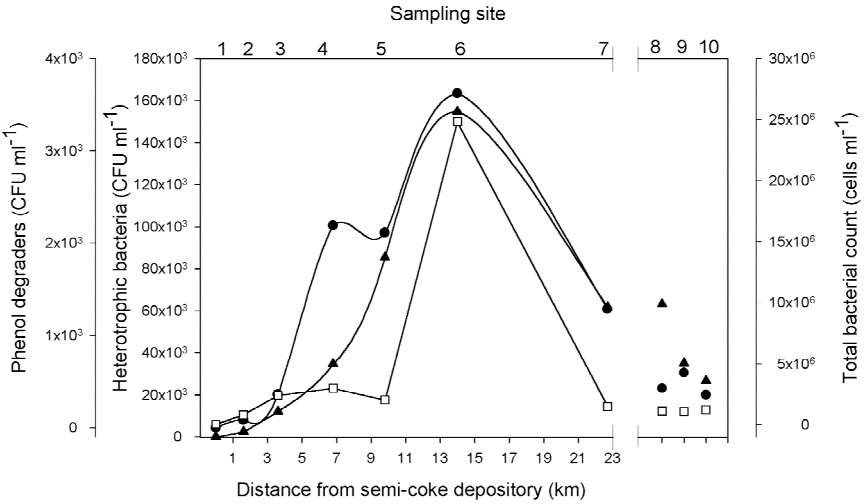

Figure 6. (Paper II, Fig. 3). Spatial dynamics of total bacterial count (□), heterotrophic bacteria (▲) and phenol degraders (●) in the sampling sites (geometric means from the period 01.05 to 06.09.1995, n=6). For sampling sites No 1–7 are shown distance from semi-coke depository (lower x-axis).

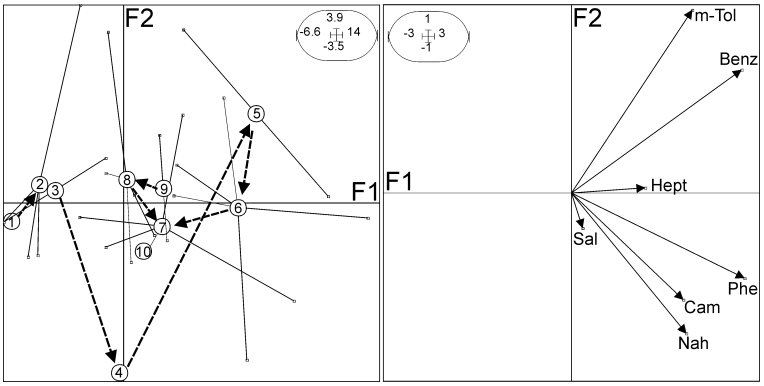

Figure 7. (Paper II, Fig. 4)**.** Ordination of the sampling sites based on Principal Component Analysis (PCA) of the abundance of biodegratative bacteria groups. A – Sampling sites along F1xF2 plane. Each sampling site is represented by the centroid of the sample scores for given site during the study period. Dashed lines indicate the flow direction between sampling sites. B – Correlation of the bacterial functional groups with two first axes (F1×F2) of the PCA. The first and second principal components describe 78.1% and 7.2% of the overall data variation, respectively. Bacterial groups according to the growth substrate: Benz – benzoate, Cam – camphor, Hep- heptane, m-Tol – *m*-toluate, Nah- naphthalene, Phe – phenol, Sal – salicylate.

Multivariate analysis of biodegradative bacteria data designated the sites into a highly polluted group (Sites No. 1, 2, 3), and an unpolluted group (Sites No. 8, 9, 10) (Fig. 7). Kohtla River sites No. 5 and 6 are clearly distinguishable from the other sites. A strong correlation was found between the first principal component and the number of lipolytic bacteria ($r=0.90$, $p<0.01$), whereas the relationship with the number of denitrifiers was weaker ($r=0.72$, $p<0.05$). According to the multivariate randomisation test, the statistical differences between sampling sites were highly significant ($p<0.001$).

Multivariate analysis of microbiological parameters revealed the changes in the river bacterial community that can be related to pollutant exposure. In general, the PCA ordination of sampling locations of the microbiological data was similar, but finer, than that of the chemical data, consequently separating control sites from river sections affected by leachate.

The lack of temporal variability during the sampling period may reflect the strong effect of pollution to water chemical and microbiological parameters. It has been shown that perturbations in microbial community structure due to pollution exposure are comparable to changes caused by seasonal factors (Findlay *et al.,* 1995). However, in our case, a longer sampling period is needed to confirm this hypothesis. Our results confirm that chronic exposure to pollution results in altered microbial community structures in river water and sediments (Langworthy *et al.,* 1998, Lehman *et al.,* 1997, Lopez-Archilla and Amis, 1999). In perturbed ecosystems, an elevated microbial biomass and abundance is found at intermediate levels of biodegradable pollutant (subsidy effect), and abundance decreases following higher pollutant loading (stress effect). In our study, the observed pattern of all studied microbiological variables is consistent with the subsidy-stress gradient. Positive deflections in bacterial total count, number of heterotrophic bacteria and biodegraders occurred at moderately polluted river sections, while the toxic effects probably override stimulatory effects in the channel area.

4.2.3. Relationships between chemical parameters and abundance of biodegradative bacteria

The permutation test revealed that the co-structure between the river biodegradative bacterial groups and the chemical variables is highly significant ($p<0.001$). The first two eigenvalues of the co-inertia analysis account for 99.1% and 0.8% of the variance, respectively. The first factorial axis (F1) displays a pattern clearly distinguishing polluted sites from the remaining locations (Fig. 8A) and is dominated by pH, COD, ammonium and total nitrogen concentrations. The bacterial functional groups are negatively correlated with all physical and chemical factors except oxygen (Fig. 8B). Based on the response to the chemical variables, the biodegradative bacteria can be separated into two groups. The first group consists of m-toluate, benzoate and salicylate

degraders and is characterised by the higher affinity for oxygen. The abundance of these bacterial groups is negatively correlated with the COD, ammonium and total nitrogen concentrations (Fig. 8C). The second group of bacteria consists of heptane, phenol, naphthalene and camphor degraders. These bacteria tend to be more sensitive to high pH values, and less influenced by the other factors.

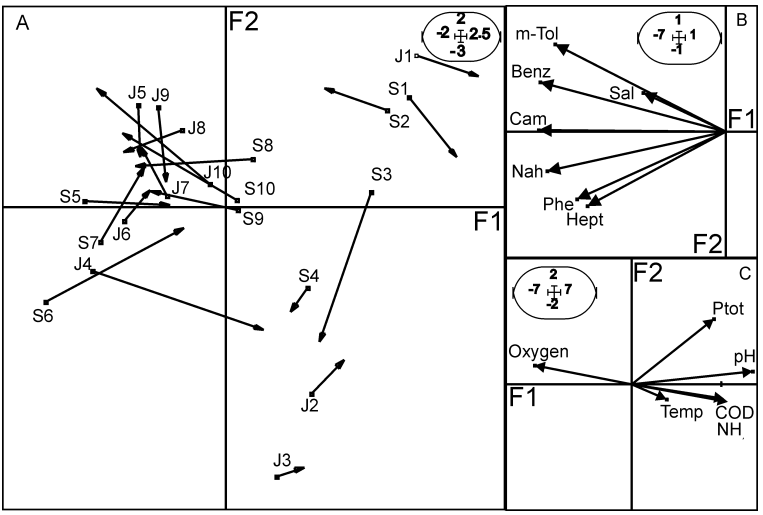

Figure 8 (Paper II, Fig. 5). Ordination diagram (F1 × F2) of the co-inertia analysis of the river chemical and microbiological data. A – Joint plot of sites; the site is identified by the first letter (J – June; S – September) and sampling site number. The beginning of arrow is the position of the sampling site according to chemical data; end of the arrow is position of the sampling site according to microbiological data. B – Biplot of the bacterial groups. C – Biplot of the chemical variables. The direction of arrow reflects the direction of maximum change of each variable; the longer the arrow, the grater its influence on bacterial groups.

The outcome of co-inertia analysis is in concordance with our earlier findings, which underscored the importance of oxygen as a positive factor and high pH as negative factor for river heterotrophic bacterial communities (Talpsep *et al.*, 1997). In addition to these two parameters, the activity of the indigenous microbial community in the channel area may be inhibited by factors that were not measured, but have a similar spatial pattern with pH or were not included in the co-inertia analysis due to missing values. Most phenol degraders, for example, have a lower tolerance to phenolic loading than other species groups which are unable to degrade phenol (Whitley *et al.,* 2001). Therefore, the very high phenol concentrations at the beginning of the channel may be toxic to degradative bacteria. Another important group of variables not included in our

study is sulphuric compounds, which may contribute significantly to toxicity of semi-coke wastewater (Kahru *et al.*, 1997).

4.2.4. Possibilities for biological treatment of semi-coke leachate (Publications V and VI)

Our findings indicate that, in order to achieve an increase in the density of biodegraders and accelerate biodegradation in the channel area, the reduction of the concentration of pollutants in wastewater prior to its discharge to the channel is required. The most feasible methods for treatment of leachate from oil-shale semi-coke heaps can be an aerated lagooning system (Orupõld *et al.,* 2000), and possibly constructed wetlands. The last option is especially promising, as the wetland system's treatment efficiency may be up to 99% for phenol (Polprasert *et al.,* 1996) and 90% for oil products (Salmon *et al.,* 1998). If more recalcitrant compounds are present in wastewater the system performance could be enhanced by the addition of well-adapted, active degradative bacteria (Groudeva *et al.* 2001). Constructed wetlands have been successfully used for treatment of petroleum industry effluents (Guodong *et al.,* 2002). While designing the constructed wetland for semi-coke leachate treatment it should be taken into account that highest phenol degradation is achieved in aerobic conditions, but COD reduction occurs also under anaerobic conditions and is coupled to sulfate reduction (Kettunen *et al.,* 1996). Thus, the constructed wetland system offering anaerobic-aerobic compartments should be optimal for semi-coke leachate treatment. The performance of passive treatment systems can be enhanced if amount and toxicity of wastewater from semi-coke depository is reduced. One possibility to achieve this aim is to utilize integrated environmental biotechnology approach for remediation of oil shale solid waste dump area. The goals of integrated approach can be accomplish through sequential application of phytotechnology and bioaugmentation.

During the years 2001–2003 we performed laboratory and field experiments in order to assess the suitability of phytotechnological approach, which includes phytoremediation in combination with bioaugmentation for remediation of semi-coke dump area. The chemical analysis of soil samples showed impact of the plant treatment on degradation rate of pollutants. Within a 16 months period starting from the establishment of test plots in July 2001, the concentration of volatile phenols was reduced by 30 to 50%, the concentration of oil products nearly 3 times (from 310 mg kg^{-1} to 120 mg kg^{-1}), and the total content of organic carbon decreased by 10 to 30 g per kg (from 18% to 15%). The best results were obtained on the plots with peat amendment and pre-grown lawn with the highest root density in semi-coke. In upper layer samples (0–10 cm) the reduction of oil products and phenols was even bigger being in the range from 83% to 98%.

Bacterial biomass consisting of three bacterial strains was applied to three experimental plots in June 2002. Within a three months period the concentration of residual shale oil in semi-coke decreased by 13.6% to 53.6% at plots treated with bacterial biomass compared to untreated parts of experimental plots. The effect of bioaugmentation could be attributed either to activity of introduced bacterial strains or to horizontal transfer of biodegradative genes. The genetic information encoding degradation of pollutants is transferred from inoculated strains to indigenous bacterial populations by catabolic genetic elements. Introduced bacterial strains may also increase root biomass, length and/or exudation leading in this way to enhanced rhizosphere bioremediation. In our experiments we also observed the increase in root biomass and length on the plots amended with bacterial biomass, which in turn may led to enhanced rhizodegradation.

Partial fragments of gene for the largest subunit of multicomponent phenol hydroxylases (LmPHs) were amplified from total DNA, separated by denaturing gradient gel electrophoresis (DGGE) and sequenced. Obtained sequences were compared with published sequences. In the plots with plants dominated two different multicomponent phenol hydroxylases (LmPH) belonging to low- and moderate K_s kinetics groups, indicating more efficient degradation of aromatics at these plots. There was only one dominant LmPH at untreated plot, belonging to high-Ks group.

From the semi-coke samples the number of aerobic heterotrophic and phenol-degrading bacteria was determined. The number of phenol-degrading bacteria increased by order of magnitude, while the number of heterotrophic aerobic bacteria remained on the same level compared to the untreated plot (Table 2 in Paper **VI**). Samples from the second year (2002) showed lower values of aerobic heterotrophic bacteria, which could be due to extremely dry vegetation period. The general trend was the increase of proportion of bio-degradable bacterial numbers within microbial community due to the treatment. Highest values for all measured microbiological parameters were found in rhizosphere samples. While bacterial total numbers increased by order of magnitude compared to control, the number of phenol-degrading bacteria was more than 100 times higher in the rhizospheric soil. Addition of bacterial biomass to semi-coke resulted in increase both in absolute number (up to 7.8×10^6 CFU g^{-1}) and relative abundance (up to 30%) of phenol-degrading bacteria in the studied samples. The highest values for microbial activity and diversity measured with Biolog EcoPlates were recorded in rhizosphere samples.

From four tested grass species *Lolim perenne* (perennial ryegrass), *Poa pratensis* (Kentucky bluegrass) and *Festuca rubra* (red fescue) were found growing on plots. The growth rate of plants was approximately twice higher in the case of peat and sand amendments. The mechanism of phytoremediation systems, based on rhizodegradation, is increase in microbial numbers and activity as well as altered microbial community functional structure due to

nutrient release by plants. This scenario has been suggested by many researchers (Haby and Crowley, 1996; Joner *et al.*, 2002; Siciliano *et al.*, 2003). Our data suggest that establishment of plants promoted increase of biodegradative bacterial number in semi-coke, especially in the vicinity of roots.

Our results indicate that phytoremediation and bioaugmentation could be considered as an alternative management option for remediation of oil shale solid waste. Additional beneficial side effects of the establishment of vegetation on the semi-coke deposit are reduction of leachate amount and toxicity, and surface erosion. Also, plant cover would diminish the dispersion of pollutants into adjacent area including Kohtla-Järve city by air. When semi-coke toxicity is decreased, it becomes more suitable substrate for trees, as mycorrhizal fungi of tree roots are more sensitive to pollutants compared to bacteria. The better growth of trees in turn favors the remediation of deeper layers of semi-coke as well stabilizes the soil structure.

4.3. Estimation of diversity of phenol degrading bacteria

4.3.1. Comparison of different typing methods using multivariate analysis techniques (Publication IV)

The ability to measure bacterial diversity is a prerequisite for the understanding how environmental factors affect microbial community succession and functioning in any ecosystem. Multitude of different methodological approaches has been developed for identification of bacterial isolates ranging from phenotyping to genotyping. Whatever method is applied two questions arise, first how well identifications from different systems match, and second, how to compare microbial diversity between different environments (locations, samples).

In Paper **IV** classifications derived from two commercial multitest systems were compared using the two multivariate statistical approaches, the Mantel's test and the co-inertia analysis. Applying the multivariate techniques for analysis on two microbiological data sets, each of which representing the results derived from two different identification systems, helps to understand the interrelationship between the outputs from these two systems. The Mantel's test indicated that grouping of studied strains according to the results from the two test systems was similar. This conclusion was also deduced from the co-inertia analysis. Our results indicate that for the cases involving the environmental strains, the API 20NE and the Biolog GN identification results are generally in good agreement on genus level, as derived from both the 24-h and the 48-h incubation readings. The comparison of identification results in a usual manner, i.e., by utilising the assigned species names, did not show high consensus in identification results, especially when the 24-h designations were compared on species level.

The advantage of using the co-inertia analysis (CIA) is that it offers an opportunity to analyze the tests from two identification systems simultaneously, thus allowing us to find out which tests contribute in like manner to the separation of bacterial strains in ordination. The API 20NE and the Biolog GN systems employ eight common carbon substrates, of which three of them, mannitol, N-acetyl-D-glucosamine and arabinose, varied in our study identically. The dissimilar results, obtained with the API 20NE and the Biolog GN for the same substrates, are obviously due to their different mode of operation, namely, to the pH changes by acids production from carbohydrates in case of the API 20NE, and to oxidation of substrates via electron transport chain in the case of Biolog GN.

A useful feature of the CIA is that it offers an opportunity to visualise the relationship between two sets of identification results on a joint scatter. In our case, the first co-inertia plane explains the very high percentage of variability attributable to each of the separate analyses alike, and the graphical representation of the co-inertia analysis on the joint scatter demonstrates high correlation between the two sets of identification results (Fig. 9). This graph also shows that the Biolog GN system offers a finer resolution due to its utilisation of higher number of carbon substrates, and, for that reason, is better suited for studies involving assessment of microbial diversity, as stated by Johnsen and co-workers (1996).

As an example how CIA can be extended to analysis of similarity matrices derived from phenotyping and genotyping results of bacterial strains, the Procrustean co-inertia analysis (PCIA) for the linking of multivariate datasets was used (Dray *et al.,* 2003b). Analysed data set consists of subset of 26 bacterial strains from Paper **IV**. Biolog identification was performed as described in Paper **IV**. In addition genotyping, namely randomly amplified polymorphic DNA (RAPD), was carried out using two primers (AP12: 5' CGGCCCCTGC 3', and C06: 5' GAACGGACTC 3') according to protocol from Harry *et al.,* 2001. Obtained RAPD profiles were analysed with GelCompar software (vers. 4.0: Applied Math, Kortrijk, Belgia) and saved as similarity matrices. Computations and graphical displays of PCIA were obtained using the ADE-4 package (Thioulouse *et al.,* 1997) and PROTEST software (Jackson, 1995).

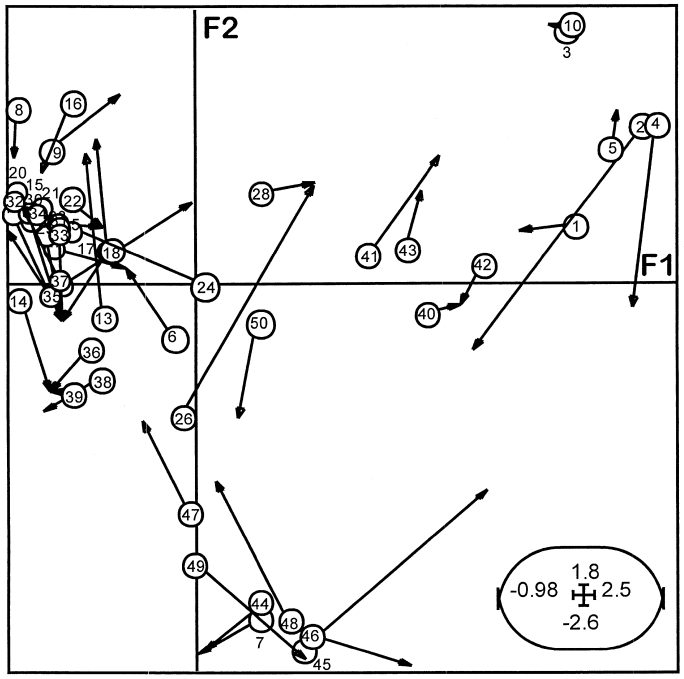

Figure 9 (Paper IV, Fig. 1). Comparison of the ordinations of studied bacterial strains resulting from co-inertia analysis using standardised coordinates. For each strain, the arrow links the position of strains resulting from the new ordination given by Biolog GN data to the position resulting from the new ordination of API 20NE data. Strain numbers are listed in Table 1 in Paper **IV**.

A barplot of singular values indicated that PCIA identifies a two-axes structure (Fig. 10). The fit between the two tables is good (m^2=0.81; goodness-of-fit statistic of Procrustean analysis) and statistically highly significant (P<0.0001). Along first axis *P. mendocina* and *P. putida* strains are separated from *P. fluorescens* biovars. Second PCIA axis depicts differences between *P. fluorescens* biovars. PCIA joint scatter indicates that Biolog systems distinguishes clearly P. *fluorescens* biovar C from the rest of P. *fluorescens* biovars, while RAPD analysis emphasis differences between P. *fluorescens* biovars A and G. This plot also suggests that strain PC17 identified by Biolog as *P. corrugata* could more likely assigned to species *P. mendocina.*

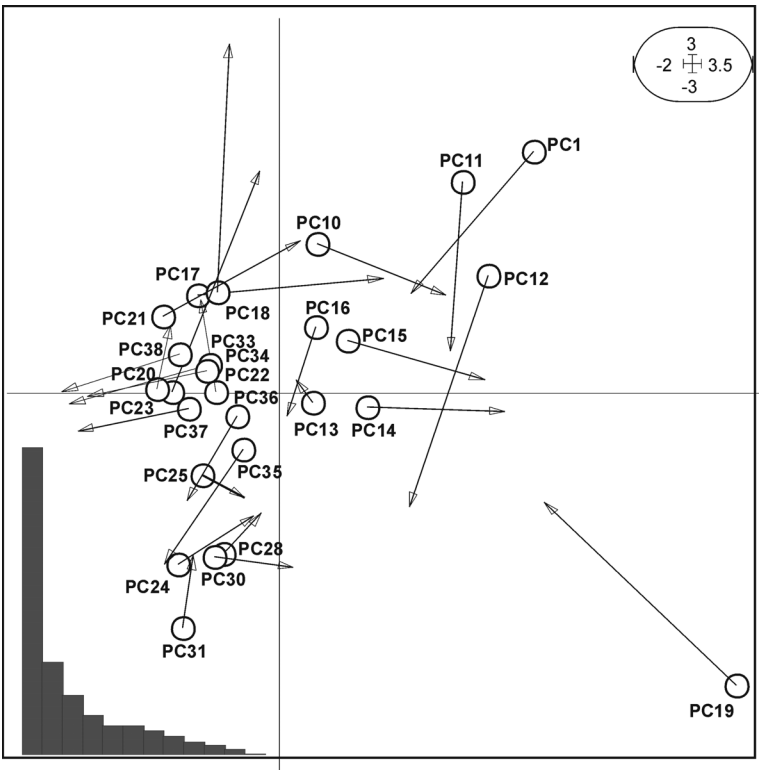

Figure 10. Procrustean co-inertia analysis of bacterial strains' phenotyping and genotyping data. Arrows are residuals between scores obtained from the Biolog phenotyping data matrix and the RAPD genotyping data matrix. Barplot of singular values is indicated.

Table 2. Strain names and species designation according to Biolog GN identification system

Species designation	**Strain name**
Pseudomonas mendocina	PC1, PC10, PC11, PC12
Pseudomonas putida A1	PC13, PC14, PC15, PC16
Pseudomonas corrugata	PC17
Pseudomonas fluorescens C	PC18, PC24, PC25, PC28, PC30, PC31
Acinetobacter genospecies 15	PC19
Pseudomonas fluorescens A	PC20, PC21, PC22, PC23
Pseudomonas fluorescens G	PC33, PC34, PC35, PC36, PC37, PC38

The two multivariate statistical methods employed in the current study both permit statistical testing of the relationship between two data sets, but differ in estimation of factors behind this relationship. The Mantel's test, being a matrix approach designed for analysis of relationships between two identification systems, allows only to compare distance matrices derived from two identification systems, and not to evaluate the contribution from each individual test to the differentiation of bacterial strains. This method is appropriate whenever one or both of the data sets present themselves as resemblance matrices. Since the co-inertia analysis establishes a linkage between several standard methods of analysis, such as principal component analysis, correspondence analysis, and multiple correspondence analysis, it is a good computational tool for exploration of relationships between various multivariate data sets in microbiological research.

4.3.2. Taxonomic diversity of phenol degrading bacteria (Publication III)

The majority of isolated phenol-utilizing bacteria belonged to the genus *Pseudomonas* (83.2%). In addition to pseudomonads the few representatives of species from genera *Stenotrophomonas* and *Acinetobacter* were detected in the channel area water samples. Figure 11 shows how sampling sites differ with respect to their species composition of phenol-utilizing bacteria taxonomic composition. Species composition changes gradually along pollution gradient being rather similar in the channel (sites No. 2, 3, 4), and exhibiting larger shifts in the Purtse and Kohtla Rivers (sites No. 5, 6, 7). The main shift in species composition along pollution gradient occurs due to increase in the relative abundance of species of *P. fluorescens* biotypes G and B, and *P. putida* biotype A1 in less polluted river locations. In the channel water with highest pollution load dominant phenol-degrading species were *P. fluorescens* and *P. mendocina.*

The dominance of genus *Pseudomonas* species among studied isolates is consistent with their widespread distribution in water and soil polluted by phenolic compounds (Suyama *et al.,* 1998**,** Sarand *et al.,* 1998, Qureshi and Purohit, 2002). Even in the pristine soils majority of isolated phenol degrading bacteria were from genus Pseudomonas (Koutny *et al.,* 2003).

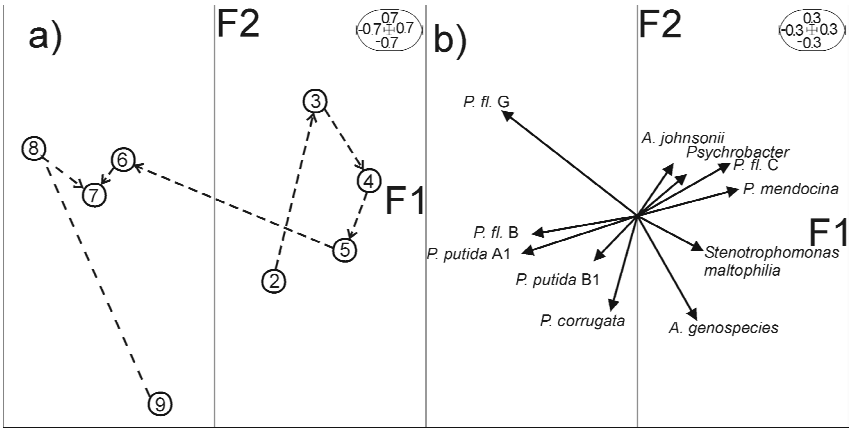

Figure 11 (Paper III, Fig. 5)**.** Ordination of sampling sites based on Principal Component Analysis (PCA) of the relative abundance of phenol-degrading bacteria taxa. **a)** Sampling sites along F1xF2 plane. Dashed lines indicate the flow direction between sampling sites. **b)** Relationship of the relative abundance of bacterial taxa with two first axes (F1×F2) of the PCA. The first and second principal components describe 40.4% and 23.7% of the overall data variation, respectively.

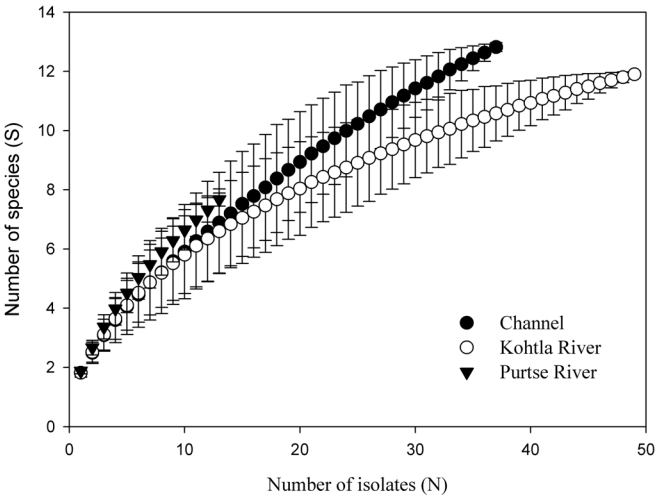

Figure 12. Rarefaction curves for phenol degrading bacteria isolates from three studied river system sections. The expected number of species (S) is plotted versus the number of isolates (N). The error bars represent 95% confidence intervals.

In order to compare impact of pollution on diversity of phenol degraders, several diversity measures were applied. Firstly, species accumulation curves

were constructed using program Analytic Rarefaction 1.3 (http://www.uga.edu/~strata/software/AnRareReadme.html). Mean accumulation curves obtained by randomizing the input order of isolates 100 times indicate that in case of the Kohtla River the richness of phenol degraders is well described, but this is not the case for the channel area, as the respective accumulation curve does not plateau (Fig. 12). The obtained accumulation curves were extrapolated by fitting two asymptotic richness estimators, the negative exponential function and the two-parameter hyperbola. These richness estimators rank the sampling locations in species richness in following decreasing order: channel, Kohtla and Purtse Rivers (Table 3).

In addition to accumulation curves a non-parametric estimator Chao I was calculated using EstimateS programme (version 6.0, R.K. Colwell, Dept. of Ecology and Evolutionary Biology, University of Connecticut [http://viceroy.eeb.uconn.edu/estimates]). This estimator was stable only in case of the Kohtla River, indicating species richness value (S*) 16, that is close to the estimate from two-parameter hyperbola model (Table 3). General richness estimator, the Shannon index does not distinguish channel and the Kohtla River, but is lower in case of the Purtse River (Table 3). The log series index (α), one of universal diversity statistics discriminates well between sampling locations, indicating higher diversity in channel area and Kohtla River compared to Purtse River.

Table 3. Diversity indices for phenol degraders.

Diversity measure	Channel	Kohtla River	Purtse River
Recorded species (S)	13	12	8
Richness estimator (S*)			
Negative exponential function	14.5±0.5	11.8±0.2	8.4±0.4
Two-parameter hyperbola	20.7±0.8	15.4±0.3	11.8±0.6
Log series index (α)	6.8±0.3	4.3±0.1	3.8±0.2
Chao 1	17±6	16±8	16.0±7
Shannon index	2.0	2.0	1.9

Analysis results indicate that in case of channel and Kohtla River, the observed number of phenol degrading bacteria species (S) is not a reliable index of their richness. The instability of diversity estimates for channel and Kohtla River means that collected data for these two locations have incomplete coverage and more isolates should be sampled for achieving true richness estimates. There are many more approaches available to estimate and compare microbial diversity data, each approach having particular strengths and limitations (Bohanann and Hughes, 2003). In our study the use of combination of different approaches suggests that diversity in one of key functional groups of river selfpurification, phenol degrading bacteria is higher in polluted river sections.

5. CONCLUSIONS

Input of oil shale underground mine water into rivers influences both water chemical and microbiological parameters. The data analysis showed increased mineralization and sulfate concentration of river water due to mine water input. The bacterial communities varied in the studied rivers according to mine water input and season. There is a further need for assessment of impact of mine water discharge to rivers in northeastern Estonia as well as for research for optimal solution for treatment of this water. In order to make direct connection between the mine water input and river microbial community composition and activity, the studies on higher resolution level using molecular microbiological methods should be applied.

The study of dynamics of microbiological variables along river system receiving wastewater from oil shale chemical industry showed a suite of responses to long-term pollution exposure. The river microbial community assemblage level parameters exhibited the subsidy-stress response to pollution load. This response was reflected by increase in the overall bacterial abundance, based on heterotrophic plate counts and direct counts, as well as on the abundance of biodegradative bacterial groups, within the river sections with moderate pollution load. The presence of an abundant and diverse community of biodegraders at channel area and the Kohtla River offers to these Purtse River system section the highest intrinsic biodegradation potential.

Multivariate data analysis methods (PCA and co-inertia analysis) proved to be effective tools for analysing and displaying the river chemical and microbiological data sets. The application of different multivariate methods allowed us not only to differentiate polluted river sections, but also to assess the impact of pollution on the river microbial community. This information is essential because the biological detoxification of pollutants is highly dependent on the abundance and structure of both river autochthonous microbial communities as well as on the remediating bacterial consortium.

An evaluation of river chemical and microbiological data suggests that the ambient natural attenuation mechanisms only partly eliminate pollutants originating from oil shale chemical industry from river water, and sufficient reduction of more recalcitrant compounds could be achieved through the reduction of pollution load from the oil-shale chemical industry into the Purtse River system.

The results of our study indicate that passive treatment systems could be most appropriate option for treatment of mine water and leachate from semi-coke depository prior release of this water into rivers. In case of semi-coke leachate constructed wetlands could be used to upgrade the quality of leachate to an acceptable level prior release into rivers. For treatment of mine water a low cost and energy-efficient method should be worked out.

Our findings support the prospect to use integrated environmental bio-technology approach for remediation of semi-coke dump area. The goals of integrated approach could be achieved through sequential application of phyto-technology, bioaugmentation, and constructed wetlands.

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SUMMARY IN ESTONIAN

Põlevkivitööstuse heitveed: mõju jõgede mikroobikooslusele ja bioremediatsiooni võimalused

Eesti peamised tahkete jäätmete tootjad on põlevkivi kaevandamine ning põlevkivikeemia ja -energiatööstus. Eestis töödeldakse termiliselt aastas kuni 1,4 miljonit tonni põlevkivi. Üle 70 aasta pikkuse ajalooga põlevkivi termiline töötlemine on põlevkivikeemiatööstuse ettevõtete ümbruses tekitanud ülisuured poolkoksi mäed, mis katavad umbes 200 ha suuruse pindala ning sisaldavad kuni 100 miljonit tonni tahkeid jäätmeid. Tahked poolkoksijäätmed sisaldavad mitmeid orgaanilisi ja anorgaanilisi ühendeid (fenoolsed ühendid, õliproduktid, väävliühendid), ladustusaladelt pärinevad nõrgveed sisaldavad aga suures koguses fenoole, kresoole, dimetüülfenoole ja resortsinoole. Poolkoksi ladustusaladelt pärinev vedel reostus ohustab pinnavett ning põhjavett.

Põlevkivikaevandustest väljapumbatav vesi juhitakse jõgedesse, mille kaudu ta voolab merre või Peipsi järve. Aastas varieerub kaevandusvee kogus vahemikus 200–250 mln m^3, millest ligikaudu 170 mln m^3 puhastatakse osaliselt enne looduslikesse veekogudesse juhtimist settebasseinides. Kaevandusvett iseloomustab võrreldes loodusliku veega suurem sulfaatide, kloriidide, kaltsiumi, raua ja karbonaatide sisaldus ning lisaks sellele veel fenoolsete ühendite ja õlijääkide olemasolu.

Käesoleva töö eesmärkideks oli uurida kaevandusvee ja poolkoksi ladestusala nõrgvee mõju Kirde-Eesti jõgede mikroobikooslusele ning biodegradatsiooni kiirust ja biodegradatiivsete bakterite mitmekesisust saastunud jõgedes.

Aastatel 1988–1991 ja 1995 Kirde-Eesti jõgedel kogutud andmete analüüsiks kasutati mitmemõõtmelise andmeanalüüsi erinevaid meetodeid. Töö tulemusena leiti, et kaevandusvee juhtimine jõgedesse mõjutab jõevee mikrobioloogilisi näitajaid, vähendades mikroobikoosluse sesoonset muutlikust. Kuidas kaevandusvesi mõjutab jõgede mikroobikoosluse liigilist struktuuri, seda nii avavee kui setete koosluste puhul, selle kirjeldamiseks oleks vaja läbi viia täpsemaid uuringuid kasutades mikroobikoosluse molekulaarseid tüpeerimismeetodeid. Sellise uurimistöö tulemused oleksid rakendatavad senisest paremate kaevandusvee puhastussüsteemide välja töötamisel.

Poolkoksi ladestusalalt pärineva nõrgvee juhtimine Purtse jõe vesikonna jõgedesse ja ojadesse mõjutas vee mikroobikooslust. Bakterite üldarv ja biodegradatiivsete bakterite arvukus oli suurim mõõdukalt saastunud jõelõikudes, samuti iseloomustas saastunud jõelõike suur fenoolilagundavate bakterite liigiline mitmekesisus.

Saastunud keskkonna mikroobikoosluste uurimise üheks eesmärgiks on ennustada, kuidas saasteainete looduslik lagundamine ehk biodegradatsioon võiks antud kohas toimida. Saadud andmeid on võimalik rakendada bioremediatsiooni meetodite väljatöötamisel ja optimeerimisel. Kogutud andmete

analüüs näitas, et Purtse jõe vesikonna jõgedes toimub biodegradatsiooni vahendusel ainult osaliselt poolkoksimägedelt pärineva saasteainete lagundamine ning suurema puhastusefektiivsuse saavutamiseks on vaja vähendada jõgedesse jõudvat saasteainete kogust.

Poolkoksi ladestusalalt pärineva reostuse mõju Kirde-Eesti jõgedele on võimalik vähendada kasutades integreeritult keskkonna biotehnoogilisi lahendusi. Töö tulemuste põhjal võib teha järelduse, et passiivsed heitvee puhastussüsteemid nagu märgala puhastid ja pinnasfiltrid oleksid sobilikud poolkoksimägede nõrgvee puhastamiseks enne selle vee jõgedesse juhtimist. Kohtla-Järve poolkoksimägedel läbi viidud katsete tulemused näitavad, et taimestikuga katmine (fütoremediatsioon) koos biodegradatiivsete mikroobide lisamisega (bioaugmentatsioon) on sobivad lahendused saasteainete biodegradatsiooni kiirendamiseks poolkoksis, mille abil on võimalik vähendada ladestusalalt pärineva nõrgvee kogust ja saasteinete kontsentratsiooni selles vees.

ACKNOWLEDGMENTS

To my supervisor, Professor Ain Heinaru, I am deeply grateful for his scientific guidance and support, and especially for encouraging my interest in application mathematics and statistics in the field of environmental microbiology. I would like to thank Prof. John Birks from the University of Bergen, Norway who give me opportunity to participate in his interdisciplinary group and introduced me to the field of environmental statistics and modeling. My thanks go also to Prof. Maxine Holder-Franklin from the University of Windsor, Canada for inviting me to her lab and giving me possibility to work with her.

I want to express my sincere gratitude to all of the co-authors of the papers. My thanks to go to whole present and previous colleagues from room 105 for helpful attitude and a nice atmosphere. I would like to express my sincere thanks to my good colleague and co-author Eeva Heinaru for her help and advice in microbiological methods.

Finally, my thanks go to my wife Marika and our daughters Liisa, Hanna and Saale, whose encouragement and support during my studies was invaluable.

PUBLICATIONS

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1992	Course on Biostatistics (Turku University, Finland)
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1994	Course on Limnology (Erken laboratory, Sweden)
1996	Course on environmental data analysis (Bergen University, Norway)
1997	Windsor University (Canada), Environmental Microbiology Research Laboratory
1997	Czech Republic — NATO Advanced Science Institute, course "Bioavailability of xenobiotics in environment"
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Professional employment

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