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FUNCTIONAL DYNAMICS OF MICROBIAL POPULATIONS IN WATERS CONTAMINATED WITH PHENOLIC LEACHATE

E. TALPSEP, E. HEINARU
J. TRUU, T. LAHT
A. HEINARU

Institute of Molecular and Cell Biology,
Tartu University
Tartu, Estonia

H. WAND

Department of Biotechnology,
University of Leipzig
Leipzig, Germany

U. STOTTMEISTER

Centre for Environmental Research (UFZ) Leipzig-Halle LTD.,
Department of Remediation Research
Leipzig, Germany

Spatial dynamics, abundance and the biodegradation potential of aquatic microbial communities were studied during the period from November 1993 until September 1995 in the Kohtla-Järve region of north-eastern Estonia. General trends in bacterial population density profiles indicated that the microbial community and the heterotrophic, lipolytic, denitrifying bacteria and biodegradative bacteria (phenol, m-toluate, benzoate, salicylate, camphor, naphthalene and heptane degrading bacteria) were strongly inhibited in the highly polluted waters with oil shale ash leachate effluent and the bacterial numbers increased as the pollution load decreased. Microbial communities revealed significant adaptation to phenolic compounds in the environment, particularly the phenol and benzoate degraders. Distribution of phenol degraders showed remarkable similarity with the heterotrophic, lipolytic and denitrifying bacterial distribution pattern. The biodegradation potential rose throughout the rivers Kohtla and Purtse revealing a high natural selfpurification process. Correlation of environmental variables and bacterial groups was shown. Substrate utilization patterns as recorded with the BIOLOG GN system was used to assess metabolic dynamics of microbial communities and classifying the sampling sites according to their degradative potential.

Introduction

Increases in the amounts and variety of chemical pollutants introduced into the environment, recognition of the importance of microorganisms in the attenuation and complete biodegradation of these compounds in nature have stimulated interest in microbiological research of aquatic ecosystems. The water ecosystems most frequently chosen for examination have tended to be marine systems [1, 2] lakes and ponds [3, 4] and estuaries [5, 6]. Few studies of microbial ecology on the population level have been devoted to rivers [7-9]. Although the role of natural biodegradation and nutrient regeneration in river systems is well known [10], detailed population studies of the free living bacteria are restricted due to methodical difficulties of investigating microbial consortium in rivers where water is flowing and changing continuously.

In describing density variations occurring in native aquatic populations and attempting to correlate with various environmental factors (oxygen, temperature, pH, nutrients, COD) spatial, temporal and geographic factors must also be considered [11]. Jones [3] showed that temporal (seasonal) studies can be difficult to interpret particularly because of inappropriate sampling frequencies. However Jones and coworkers have suggested that if a greater understanding of bacterial population dynamics is to be achieved, then long term surveys with long inter-sample intervals (over a period of a week) of the whole population might be most profitable. Alternatively it might be also profitable to adopt an autecological approach and consider changes in a particular species or functional groups of bacteria. This could include responses to chemical components in the environment both in the long and short term.

The application of the community-level approach to assays of microbial content would provide a more sensitive and ecologically meaningful measure of heterotrophic microbial community structure [12]. A community-level assay based on carbon source characters requires rapid multiple assays of carbon source utilization. Garland and Mills [12] showed that direct incubations of environmental samples in BIOLOG microplates with 95 different substrates produce utilization patterns, which are useful in classification and characterization of heterotrophic microbial communities.

The phenolic pollution of the rivers in north-eastern Estonia is mainly caused by the oil-shale industry. The drainage leachate from the ash dump formed as a by-product of oil-shale processing and chemical industry waters (up to 7200-8000 m^3 per day) are discharged without treatment via the Channel system to the rivers Kohtla and Purtse and finally into the Baltic Sea [13]. This creates a serious pollution problem. The existing water cleaning station near Kohtla-Järve needs complete rebuilding for cleaning leachate pollution but this approach is nowadays

too expensive for Estonia. That is why there is a need to improve natural biodegradation activity in the channel and rivers through which the pollution flows to the Baltic Sea.

Our goal was to assess the effect of pollution on the river water by investigating 10 different functional groups of bacteria. Particular emphasis has been given to the dynamics of biodegradation activity of bacterioplankton.

This study also investigates multiple functional responses of the whole environmental samples to characterize functional dynamics of microbial communities and if substrate utilization patterns are suitable for monitoring variations of the composition in microbial communities, caused and influenced by pollution with phenolic leachate. Spatial variations in microbial communities in these two rivers are reported.

Material and Methods

Sampling Area and Collection of Water Probes

The study area is situated near the Baltic Sea in north-eastern of Estonia (Fig. 1).

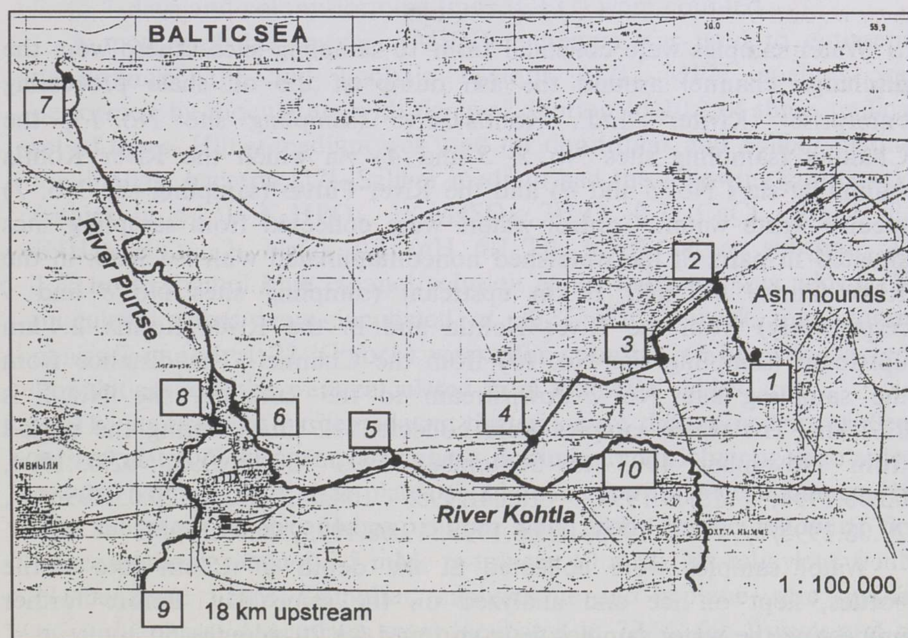

Fig. 1. The map of the sampling area. Legend: *1* - plant area; *2* - Channel (beginning); *3* - Channel (middle); *4* - Channel (end); *5* - Kohtla (+Channel); *6* - Kohtla (end); *7* - Purtse (+Kohtla); *8* - Purtse (control I); *9* - Purtse (control II); *10* - Kohtla (control)

Table 1. **General Chemical, Physical and Biological Data**

Parameter	Sampling site				
	2 Channel (beginning)	*6* Kohtla (+Channel)	*7* Purtse (+Kohtla)	*8* Purtse (control I)	*10* Kohtla (control)
Temperature, °C	7.3	6.8	7.2	7.2	6.9
Dissolved oxygen, $mgO_2\ l^{-1}$	nd.	8.4	8.7	9.1	7.7
pH	9.4	7.6	7.6	7.5	7.6
Dry material, $mg\ l^{-1}$	3297	817	774	762	820
Total carbon, $mg\ l^{-1}$	2349	591	536	546	560
Suspended solids, $mg\ l^{-1}$	153.9	8.6	6.3	5.3	4.4
BOD_7, $mgO_2\ l^{-1}$	323.9	13.6	4.3	2.9	2.1
BOD_{21}, $mgO_2\ l^{-1}$	556.3	21.1	8.5	7.5	3.5
COD, $mgO_2\ l^{-1}$	1553	123.2	76.8	46.8	34.7
NH_4, $mg\ l^{-1}$	13.7	1.2	0.7	0.5	0.5
Total N, $mg\ l^{-1}$	25.8	4.0	2.3	1.4	1.7
Total P, $mg\ l^{-1}$	0.26	0.04	0.04	0.03	0.02
Cl^-, $mg\ l^{-1}$	854	100	47	34	96
SO_4^{2-}, $mg\ l^{-1}$	600	189	188	170	76
S^{2-}, $mg\ l^{-1}$	183.0	3.2	2.6	1.1	0.8
Oil products, $mg\ l^{-1}$	0.9	1.5	0.4	0.3	0.3
Volatile phenols, $mg\ l^{-1}$	31.600	1.140	0.050	0.030	0.001
Total phenols, $mg\ l^{-1}$	73.50	5.30	0.80	0.80	0.15

Note: nd. - not determined.

Water samples were collected from 10 sampling sites. These were the discharge channel around the ash dump of the oil shale processing enterprise "Kiviter" Ltd., Kohtla-Järve (sampling site No. *1*), the Channel (sampling sites No. *2*, *3* and *4*) via which the River Kohtla (sampling sites No. *5* and *6*) and the River Purtse (sampling site No. *7*) receive waste water. Control waters were collected from sampling sites situated in parts of the watershed noncontaminated with leachate: in the River Purtse 0.5 and 18 km upstream (sampling sites No. *8* and *9* respectively) from the River Kohtla and in the River Kohtla 2.4 km upstream (sampling site No. *10*) from the Channel. The distance from the sampling site No. *1* downstream to the sampling site No. *7* is 22.1 km. The water samples were collected 12 times during the period from November 1993 to September 1995 (30.11.1993, 02.03.1994, 02.06.1994, 15.09.1994, 18.04.1995, 08.05.1995, 29.05.1995/*a*/, 29.05.1995/*b*/, 30.05.1995, 02.06.1995, 19.06.1995, 06.09.1995).

Water samples were collected at the depth of 15 cm into sterile bottles, kept on ice and analyzed on the same day. Before further processing the water samples were vortexed for 20 minutes.

Chemical, Physical and Biological Analyses

General characteristics of the water samples are shown in Table 1. Data are means of 27 monthly determinations (from September 1993 to

November 1995). Analyses on temperature, dissolved oxygen, pH, dry material, total carbon, suspended solids, BOD_7, BOD_{21}, COD, ammonia nitrogen, total nitrogen, total phosphorus, Cl^-, SO_4^{2-}, S^{2-}, oil products, volatile phenols and total phenols were kindly supplied by the laboratory of the oil shale processing enterprise "Kiviter".

Acridine Orange Direct Count (AODC) of Bacteria

Samples for direct microscopic counts were preserved in 2 % formaldehyde and stored at 4 °C until analysis. The total number of bacteria was determined by the acridine orange direct count method [14]. The bacteria were counted on 0.22-μm pore size irgalan black Nuclepore polycarbonate membrane filters (Costar GmbH, Germany) using the fluorescence microscope Olympus VANOX-SAHBS (Olympus Optical Co., Ltd.).

Cultural Methods

The heterotrophic plate count (HPC) was determined by spread plate method in triplicate by R2A agar [15]. Plates were incubated at 22 °C for 5- to 7-days and colony-forming units (CFU) were counted.

A most probable number (MPN) technique was used to enumerate denitrifying [16] and lipolytic bacteria [17]. MPN analysis were performed by inoculating with decimal dilution to 10^{-5} of three replicate set of tubes. Hiltay medium was used for denitrifiers and Seliber medium for lipolytic bacteria [17]. Seliber medium had the following composition (values in g l^{-1}): K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 6H_2O$, 0.1; $(NH_4)_2HPO_4$, 2; NaCl, 0.1; pH 6.7-7.0. 6.5 % oil as substrate and 1 % brom thymol blue ethanol solution as an indicator was added. The inoculated tubes were incubated at 30 °C for 1 week for counting denitrifiers and at 22 °C for 4 weeks for counting of lipolytic bacteria. Population densities were calculated from the MPN tables.

The numbers of biodegradative bacteria were determined in triplicate sets of plates. Water samples without preliminary enrichment were spread on the M9 salts [18] agar plates supplemented with trace elements [19]. The substrates as sole carbon sources were added at the following final concentrations: phenol 2.5 mM; *m*-toluate 10 mM; Na-salicylate 5 mM; Na-benzoate 5 mM. Camphor, naphthalene and heptane were supplied in vapor phase. Agar plates were incubated at 22 °C for 1 week and the numbers of biodegradative bacteria were expressed as CFU per ml. For further studies single colonies were picked up randomly and further culturability on respective selective agar was controlled. Count of culturable colonies was expressed as cells ml^{-1}.

Determination of Substrate Utilization Pattern Using the BIOLOG System

The metabolic diversity of the water communities was analyzed by the BIOLOG GN microplates (BIOLOG, Inc.) [12] from the samples collected in September 1995. Each of the test well of microplates were inoculated with 150 µl river water sample using an 8-channel repeating pipette. After incubation of the microplates at 22 °C for 24 h and 48 h the color development was automatically recorded by a microplate reader (Labsystems Multiscan MCC/340).

Statistical analyses

BIOLOG results were analyzed with CANACO 3.12 software from Microcomputer Power, Inc. (Ithaca, N.Y.). Actual absorbance values after correction of the absorbance from the control well were used in analysis. Each substrate value for a plot was the average of three replicate plates. For the analysis by CANACO, the 95 substrates of BIOLOG GN plates were considered individual species. The indirect gradient analysis method, detrended correspondence analysis was used to visualize the relationships among samples and relate differences in carbon source utilization by microbial community to external variables. The microbial counts in the results were presented as geometric means. The microbiological and chemical data were analyzed by Spearman rank correlation analysis.

Results and Discussion

Natural Biodegradation

Many monophenolic compounds are easily degradable in nature [20, 21]. It is difficult to calculate directly the biodegradation efficiency in the Channel, the River Kohtla and the River Purtse. Fluctuations within the sampling site were significantly wide during the period of investigation and depended greatly on the weather conditions, mainly on rainfall and also on chemical industry processing conditions (data not shown). These conditions cause fluctuations in the amount and concentration of toxic organic compounds in the leachate [22]. The concentration of phenolic compounds exceeded up to 230 mg l^{-1}. Flow rates during the year are also very different. On the basis of data from the laboratory of oil shale processing enterprise "Kiviter" we calculated that the dilution rate of the phenolic effluent in the River Purtse could be up to 180 times. The average concentration of volatile phenolic compounds in the River Purtse differs 672 times from that in the beginning of the Channel (Table 1). This value indicates that in the system we investigated the natural

biodegradation is very active. Our indirect calculations showed that selfpurification of the water system investigated exceeded 85 % (data not shown). It is reasonable to believe that such active selfpurification activity could be explained by studying the microbial community and the dynamics of bacterioplankton.

Total Bacterial Count

Although total bacterial count is considered to be rather inflexible character, here the waters with drastically different leachate concentration showed different AODC values. Direct counts ranged between 1.2×10^5 and 2.4×10^7 cells ml^{-1} (Fig. 2).

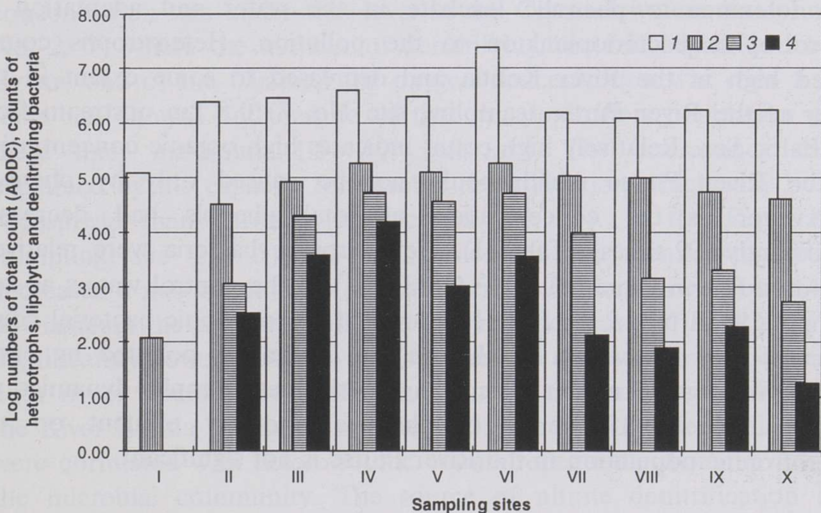

Fig. 2. Spatial dynamics of total bacterial count (AODC), heterotrophic, lipolytic and denitrifying bacteria in the sampling sites (average data from the period November 1993 until September 1995). Legend: I - plant area; II - Channel (beginning); III - Channel (middle); IV - Channel (end); V - Kohtla (+Channel); VI - Kohtla (end); VII - Purtse (+Kohtla); VIII - Purtse (control I); IX - Purtse (control II); X - Kohtla (control); *1* - AODC, cells/ml; *2* - heterotrophs, CFU/ml; *3* - lipolytic bacteria, MPN cells/ml; *4* - denitrifying bacteria, MPN cells/ml

The maximum abundance of bacterioplankton was estimated to be in the River Kohtla (sampling site No. *6*) before joining with the River Purtse (Fig. 2). Two orders of magnitudes lower was the AODC in the water of the round channel at the plant area (sampling site No. *1*) where very severe conditions exist for microflora, average pH 12.7 and high concentration of phenols [13]. AODC of the waters of the other sampling

sites did not differ significantly as the data were estimated in order 10^6. In the control waters without phenolic leachate pollution (sampling sites No. *8*, *9*, *10*) the count was not lower either.

Dynamics of Heterotrophic Bacteria

Conventional count of heterotrophic bacteria varied between 116 CFU ml^{-1} in the water of the plant area and 1.8×10^5 CFU ml^{-1} in the Channel water (Fig. 2). In the beginning of the Channel the heterotrophic count increased rapidly more than 2 orders of magnitude and as the distance from the plant area increased, the number of heterotrophs increased downstream and gained the maximum value in the water of the Channel (sampling site No. *4*). This fact indicates to the high tolerance to phenolic leachate in the water and adaptation of heterotrophic bacterioplankton to the pollution. Heterotrophs counts stayed high in the River Kohtla and decreased to some extent in the water of the River Purtse (sampling site No. 7) 0.5 km upstream from the Baltic Sea. Relatively high count indicates high organic concentration in the River Purtse which could not be caused only by phenolic wastewater as the concentration of total phenols had decreased significantly (92 times) (Table 1). Heterotrophic bacteria were relatively abundant (mean value 6.3×10^5 CFU ml^{-1}) in the control waters as well (sampling sites No. *8*, *9*, *10*). High level of heterotrophic bacterial count indicates that the waters of that region are rather polluted by easily degradable organic matter. According to the heterotrophs dynamics we suppose that the influence of chemical industry effluent on the heterotrophic population in the River Purtse is not significant.

Dynamics of Lipolytic Bacteria

The population of lipolytic bacteria revealed analogous dynamics to heterotrophs although the absolute values were different (Fig. 2). The lipolytic bacteria were counted from the channel water around the ash dump area only once. In all other samples studied the viable counts were recorded. The count of lipolytic bacteria ranged from 1.1×10^3 cells ml^{-1} up to 5.3×10^4 cells ml^{-1} in the waters contaminated. High counts were observed downstream of the Channel and the River Kohtla. The fact that lipolytic bacteria are more abundant in moderately contaminated waters might be explained by the oil products occurrence in these waters but the maximums of the lipolytic bacteria and the oil concentration do not coincide (Table 1, Fig. 2). In the River Purtse the lipolytic count decreased, which probably indicated a decrease of the concentration of the substrate. The minimum lipolytic count 540 cells ml^{-1} of noncontaminated waters appeared to be almost two magnitudes lower compared to the maximum lipolytic count. Significant differences in

bacterial counts showed the influence of the phenolic wastewater on the bacterial lipolytic population and we confirmed the biodegradation ability of lipolytic population. Further studies of isolates with lipolytic activity proved their phenol degrading ability.

Dynamics of Denitrifying Bacteria

Population of denitrifiers was less abundant than the above named functional groups both in contaminated and in noncontaminated waters. Somewhat surprisingly they revealed a similar distribution pattern (Fig. 2). Denitrification is considered to be an anaerobic process and the expression of denitrification enzymes is induced if oxygen concentration is below 5 mg l^{-1} [23]. In the studied waters, dissolved oxygen concentration was under that level in the Channel waters (sampling site No. *2*, *3*) where the number of denitrifying bacteria began to increase. By the end of the Channel the oxygen concentration was over 5 mg l^{-1} (data not shown) and despite of that the denitrifiers population gained there their maximum 1.5×10^4 cells ml^{-1}. The discrepancy can be explained by the capacity for aerobic denitrification which may be more widespread than currently appreciated [24, 25]. In the River Kohtla (sampling site No. *5*) the abundance of the denitrifying bacteria decreased a magnitude fold. A small increase in denitrifiers count 4.3 km downstream the river was followed by rapid decrease in the River Purtse. Significantly lower values were obtained in noncontaminated parts of both rivers whereas the minimum count 17 cells ml^{-1} was calculated in the River Kohtla (sampling site No. *10*). The fact that denitrifiers counts were correlated with heterotrophic counts reflects the dynamic nature of the microbial community. The source of nitrate denitrification is the external nitrate pool and *in situ* nitrate production from nitrification. Nitrate is formed due to the oxidation of ammonium which is produced by heterotrophs during mineralization of organic matter. Albeit nitrification links organic matter decomposition (ammonification) to denitrification there still exists a contradiction as nitrification is usually inhibited in the contaminated waters because of high sensitivity of nitrifiers to environmental toxicity [26].

Comparison of heterotrophs with denitrifiers and lipolytic bacteria revealed a rather similar distribution pattern although the absolute numbers were different (Fig. 2). The populations of named functional groups were adapted to the toxic compounds and maximums were detected already in the channel waters. The difference between heterotrophs and the other two groups studied was that heterotrophs counts were high in the control waters also. So the conditions for heterotrophic bacteria were favorable also in noncontaminated waters. Spatial variability of heterotrophic, lipolytic and denitrifying bacterial counts was larger than the variability of total bacterial count.

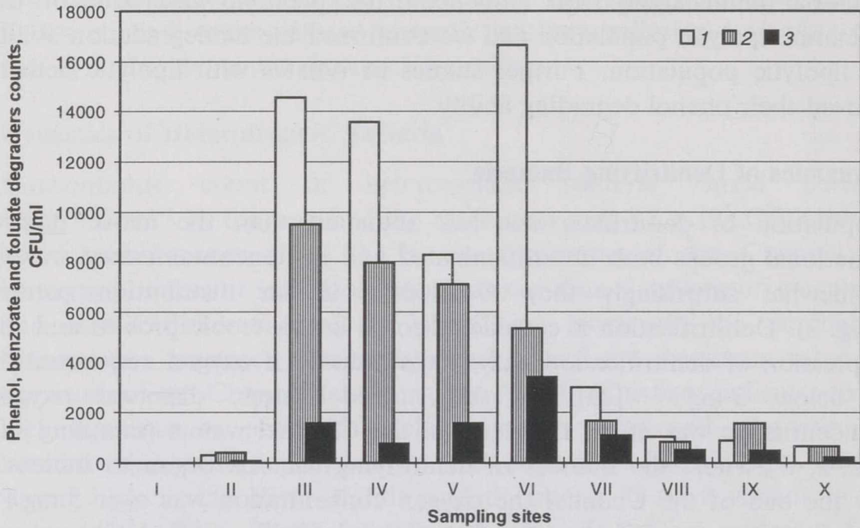

Fig. 3. Spatial dynamics of phenol, benzoate and *m*-toluate degrading bacteria in the sampling sites (average data from the period November 1993 until September 1995). Legend: I - plant area; II - Channel (beginning); III - Channel (middle); IV - Channel (end); V - Kohtla (+Channel); VI - Kohtla (end); VII - Purtse (+Kohtla); VIII - Purtse (control I); IX - Purtse (control II); X - Kohtla (control); *1* - phenol; *2* - benzoate; *3* - *m*-toluate

Dynamics of Functional Groups of Biodegradative Bacteria

The numbers of phenol, benzoate, *m*-toluate, salicylate, camphor, naphthalene and heptane degraders were determined and spatial dynamics of biodegradative bacterial populations was studied (Figures 3 and 4, Table 2). Biodegradative bacterial CFU values were expectedly the lowest in the channel around the ash heaps. Only in rare cases colony forming units of biodegradative groups were detectable but the counts did not exceed magnitude 10 during the whole sampling period despite the total microbial population density reaching 10^5. A complete absence of salicylate and toluate degrading CFU-s was registered at this sampling site. An extremely low number of culturable bacteria could be explained by the high pollution rate of this water. Continually the influence of discharged leachate effluent on the biodegradative microbial abundance was detected in the Channel waters. Despite the high concentration of toxic compounds in the beginning of the Channel all the bacterial groups studied were detectable, although CFU values were generally low. The salicylate degraders showed the minimum number (20 ml^{-1} CFU) but the other functional groups were more numerous, the numbers ranged between 91 ml^{-1} CFU of *m*-toluate degraders up to 437 ml^{-1} CFU of camphor degraders.

Table 2. **Numbers of Biodegradative Bacteria in Different Sampling Sites**

Sampling site	Growth substrate													
	Benzoate		Phenol		*m*-Toluate		Naphthalene		Salicylate		Camphor		Heptane	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1 Plant area	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0
2 Channel (beginning)	3.9	0.6	2.9	0.1	0.9	0.0	4.1	0.0	0.2	0.0	4.4	0.0	2.6	0.0
3 Channel (middle)	95.2	4.8	145.7	2.4	15.8	0.2	21.9	0.0	1.5	0.1	0.8	0.0	11.6	0.2
4 Channel (end)	79.8	15.8	135.8	10.5	7.7	0.6	88.6	0.2	1.9	0.4	87.5	0.2	45.6	0.1
5 Kohtla (+Channel)	71.1	26.1	82.8	23.4	15.6	3.2	86.2	5.0	2.0	0.3	102.4	2.3	20.6	0.1
6 Kohtla (end)	53.4	18.3	166.4	135.3	34.1	7.7	161.1	1.0	3.9	0.2	112.7	2.4	95.4	0.7
7 Purtse (+Kohtla)	16.7	12.0	30.0	12.6	11.0	1.9	41.6	1.1	2.2	0.2	45.3	0.3	15.5	0.3
8 Purtse (control I)	8.5	1.1	10.3	0.5	5.1	1.5	19.1	0.1	1.7	0.2	11.0	0.2	6.4	0.1
9 Purtse (control II)	15.9	1.2	8.7	0.7	4.8	0.4	34.9	0.1	2.4	0.3	28.4	0.4	15.5	0.5
10 Kohtla (control)	6.5	0.6	6.7	0.3	2.2	0.1	12.6	0.3	1.6	0.1	22.1	0.3	5.6	0.3

Notes: Numbers are average values from the data of 12 sampling series during November 1993 to September 1995.
Data in every experiment were means from triplicate determinations.
A - CFU $ml^{-1} \times 10^{-2}$; CFU ml^{-1} - viable count performed as colony forming units.
B - Cells $ml^{-1} \times 10^{-2}$; Cells ml^{-1} - viable count repeatedly culturable bacteria.

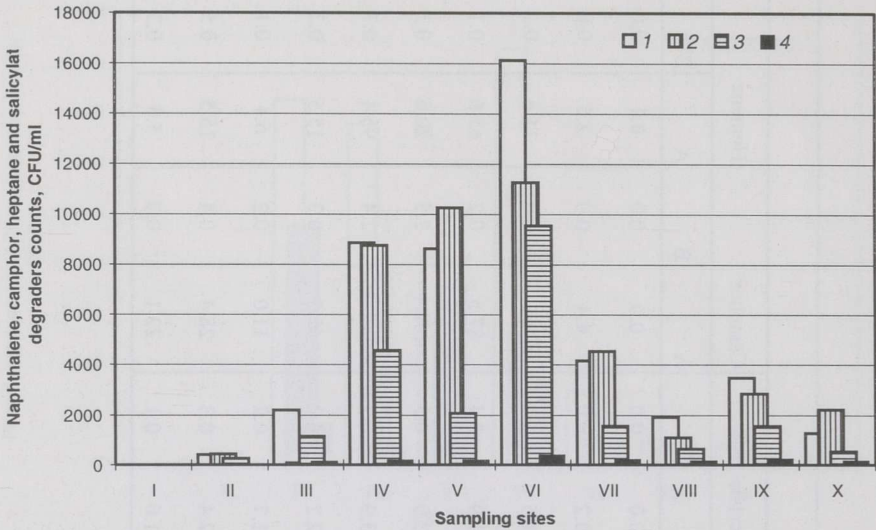

Fig. 4. Spatial dynamics of naphthalene, heptane, camphor and salicylate degrading bacteria in the sampling sites (average data from the period November 1993 until September 1995). Legend: I - plant area; II - Channel (beginning); III - Channel (middle); IV - Channel (end); V - Kohtla (+Channel); VI - Kohtla (end); VII - Purtse (+Kohtla); VIII - Purtse (control I); IX - Purtse (control II); X - Kohtla (control); *1* - naphthalene, *2* - camphor, *3* - heptane, *4* - salitcylate

Downstream of the Channel and the River Kohtla, densities of the populations of biodegradative bacteria increased as the pollution load decreased. In the middle of the Channel (sampling site No. *3*) the remarkable rise of bacterial counts reached up to magnitude 10^4 for phenol and benzoate utilizers but not for the others groups of biodegradative bacteria. A rapid rise of naphthalene, camphore and heptane degrading bacteria began at the end of the Channel. These bacterial numbers verify adaptation on a community level. It is also possible that some toxic components had already been eliminated. Surprisingly the maximum of benzoate degrading bacteria was calculated in the Channel water whereas the maximum for all other biodegraders were detected in the River Kohtla (sampling site No. *6*). Phenol degraders were the most abundant among the biodegrading bacteria and their count was registered 1.6×10^4 CFU ml^{-1}. The camphor and naphthalene degraders counts were calculated in the same order of magnitude whereas benzoate, *m*-toluate and heptane degraders numbers were one order of magnitude lower. Salicylate utilizers were the least abundant (3.8×10^2 CFU ml^{-1}). Leahy with coworkers [20] has shown that the numbers of hydrocarbon utilizing bacteria and their relative abundance in the bacterial communities increase significantly if usable

hydrocarbons are available. The increase of bacterial numbers from the Channel to the end of the River Kohtla indicates the available substrate and also the decrease of toxic components to the bacteria.

In the River Purtse biodegradative bacterial population decreased and was comparable to the bacterial abundance of non polluted waters of the studied rivers. Albeit pollution load decreased in the River Purtse, the bacterial numbers did not increase anymore but showed decline, which is probably connected with the decrease of growth substrate. Another important point is that when the River Kohtla is joining with the River Purtse, the water of the River Kohtla would be diluted about 10 times. From the September 1995 water samples we isolated 3050 fast-growing biodegradative bacterial strains and found that 1689 of them were phenol degraders. The distribution data of those phenol degrading bacteria are presented in Fig. 5. It reveals that among the biodegradative bacteria the number of phenol utilizing bacteria began to rise at the end of the Channel and reached its maximum value in the River Purtse. Although those data are indirect, they show that in the River Purtse the total number of phenol-degrading bacteria is even higher than in the River Kohtla but due to the dilution effect and low concentration of phenolic compounds the further rise of abundance of this group of bacteria stopped (Fig. 3).

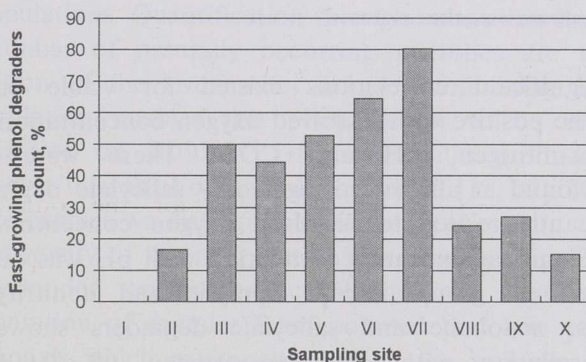

Fig. 5. Distribution of fast-growing phenol degrading bacteria among isolated biodegrading bacteria in the sampling sites (September 1995). Altogether 3050 isolates were studied. Legend: I - plant area; II - Channel (beginning); III - Channel (middle); IV - Channel (end); V - Kohtla (+Channel); VI - Kohtla (end); VII - Purtse (+Kohtla); VIII - Purtse (control I); IX - Purtse (control II); X - Kohtla (control)

Correlation Between Bacterial Groups and Environmental Factors

Bearing in mind the observations made it was attempted to draw some general conclusions on the effect of certain environmental variables (pH,

temperature, dissolved oxygen concentration, total nitrogen and ammonia-nitrogen, total phosphorus and COD) on bacterial populations as estimated by direct and viable count procedures. The Spearman rank correlation between studied bacterial populations estimates and these variables are represented in Table 3.

Table 3. **Spearman Rank Correlations of Physico-Chemical and Bacteriological Parameters**

Bacterial groups	External environmental variables					
	pH	Temperature	Dissolved oxygen	Ammonia-nitrogen	Total nitrogen	COD
Heterotrophs	-0.37*2	ns	0.31*1	-0.64*3	-0.42*1	-0.51*1
Lipolytic bacteria	ns.	ns	ns	ns	ns	ns
Denitrifying bacteria	ns	ns	ns	ns	ns	ns
Benzoate degraders	-0.36*2	ns	ns	ns	ns	ns
Phenol degraders	-0.39*2	ns	ns	ns	ns	ns
m-Toluate degraders	-0.51*3	-0.32*1	ns	-0.47*3	-0.33*1	ns
Camphore degraders	-0.63*3	ns	ns	-0.49*1	-0.45*1	ns
Naphthalene degraders	-0.65*2	ns	ns	-0.42*1	ns	ns
Heptane degraders	-0.66*3	ns	ns	-0.45*1	ns	ns
Salicylate degraders	-0.61*2	ns	0.52*3	-0.57*3	-0.33*1	-0.49*3

Notes: *1, *2, *3 - represent the significance of correlation at $P = 0.05, 0.01, 0.001$, respectively.
ns - not significant.
"-" - indicates a negative correlation.

Where significant correlations existed for viable bacteria, these appeared to be positive with dissolved oxygen concentration and negative to ammonia-nitrogen, pH and COD. There were few positive correlations found at all. Surprisingly only salicylate degrading bacteria were significantly related to dissolved oxygen concentration ($r = 0.52$, $P < 0.001$). Significant negative correlation with pH was obtained for all studied functional groups except lipolytic and denitrifying bacteria. Heterotrophs, *m*-toluate and salicylate degraders showed significant negative correlation with ammonia-nitrogen ($P < 0.001$). Salicylate degraders were additionally highly negatively correlated to COD (Table 3). Heterotrophs counts showed significant negative correlation to pH ($r = -0.37$; $P < 0.001$) and somewhat lower to COD ($r = -0.51$, $P < 0.05$) which explains the low counts in the waters of plant area. Surprisingly ammonia-nitrogen appeared highly negatively correlated to heterotrophic population ($r = -0.64$, $P < 0.0001$). Similar negative correlation with ammonia-nitrogen was obtained from nutrient rich lake waters [3]. Correlation was also obtained with dissolved oxygen concentration ($r = 0.31$, $P < 0.05$).

It is interesting to underline that phenol and benzoate degraders as well as lipolytic and denitrifying bacteria have no significant correlation

with temperature, dissolved oxygen, ammonia-nitrogen, total nitrogen and COD (Table 3). In this sense these groups of bacteria differ from heterotrophs and other groups of biodegradative bacteria. Among the biodegrading bacterial population greatest adaptation was exhibited by phenol and benzoate degrading bacteria (Figures 3 and 4). Other biodegradative bacteria are less adapted and minimal response to phenolic leachate was found in the population of salicylate degraders. Low population abundance and low variability indicates that salicylate degrading bacteria are of little importance in the studied waters. As stated already heterotrophs, lipolytic and denitrifying bacteria had significant resemblance in spatial dynamics (Fig. 2). Comparing their distribution patterns with the biodegraders patterns we find very strong analogy between phenol degraders and lipolytic and denitrifiers (Figures 2 and 3). The fact that these bacterial populations show such remarkable analogy reflects the important link between them in microbial community.

Characterization of Microbial Communities Studied on the Basis of Patterns of Community-Level Sole Carbon Source Utilization

Sole carbon source utilization was used as a functional measure at community-level in characterization of heterotrophic microbial communities. Community metabolic pattern analysis is an alternative approach to link the study of microbial community and the quantification of their constituent populations. Quantification through cultivation has limitations as a great number of naturally occurring microbes are uncultivable. Direct incubation of the river water samples containing intact whole microbial population enables this phenomenon to be encompassed. Substrate utilization on BIOLOG GN microplates produced response patterns to resolve microbial community structure [12, 27]. According to these metabolic profiles we distinguished sampling sites.

Metabolic diversity of the river waters was strongly impacted by toxic chemicals. Detrended correspondence analysis of the river samples revealed a separation of samples from polluted and non polluted sites along first CA axis which explained 21 % of the variance in the data (Fig. 6). The second DCA axis which explained only 12 % of the variance of the data was related to community variability within heavily polluted river sites. Long distance between samples from strongly polluted and non polluted sites along the first DCA axis indicates that these sites had few common metabolized substrates. The figure explicates differences between microbial communities from different sampling sites. According to the analysis the polluted sites were *1*, *2* and *3*. The next polluted site appeared to be No. *8* but this is obviously a discrepancy as the waters of that sampling site are not polluted with phenolic leachate. It is still possible that some other kind of wastes have been received.

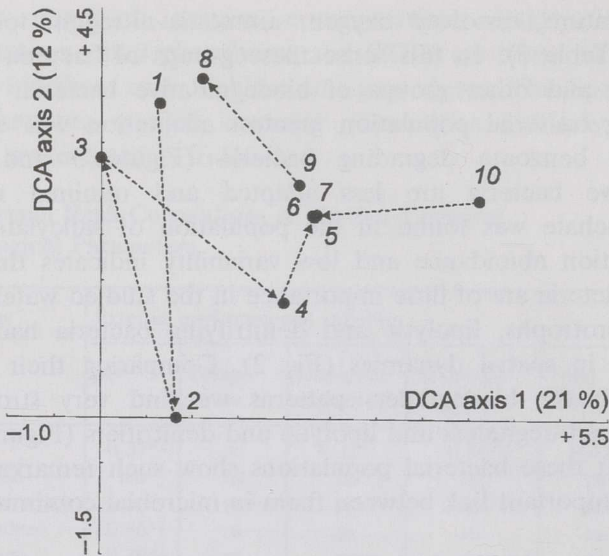

Fig. 6. Results of detrended correspondence analysis of river water samples. Data represent the coordinate scores for the first two ordination axes. Arrows show the flow direction between sampling sites

Comparison of bacterial counts from three control sites showed that the counts were the highest there which also may indicate to some additional waste. Community structure of sampling sites *5*, *6* and *7* formed one group and thus revealed high similarity. Bacterial counts support the fact, with the exception that the bacterial abundance was generally an order lower in the delta of the River Purtse (sampling site No. *7*). Relatively close to the former group was the microbial community of the sampling site *4*. Although population density there was lower, selfpurification had already been induced and the community structure revealed similarity with the microbial community of the River Kohtla. The most clean water appeared to be in the unpolluted part of the River Kohtla and the community structure differs from the previous communities. This was also supported by the viable microbial counts.

We related DCA analysis axis to the external environmental variables. The first DCA axis was positively correlated with water oxygen concentration ($r = 0.60$) and negatively with pH, COD and total phosphorus ($r = 0.60$-0.63). There was no statistically significant correlation between second DCA axis and environmental variables.

Our results indicated that BIOLOG community assay would be a useful tool for classifying river water microbial communities but this promise can be fulfilled only through careful evaluation of the obtained multivariate pattern using different numerical analysis methods. A long period of oil shale industrial waste water pollution has induced natural

selection of microbial population that can, not only survive in these extreme conditions, but also effectively biodegrade phenolic components of the leachate.

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REFERENCES

1. *Ammermann J. W., Fuhrman J. A., Hagström A., Azam F.* Bacterioplankton growth in sea water. I. Growth kinetics and cellular characteristics in sea water cultures // Mar. Ecol. Prog. Ser. 1984. V. 18. P. 31-39.
2. *Yoon W. B., Rosson R. A.* Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to dial variation in seawater turbidity // Appl. Environ. Microbiol. 1990. V. 56. P. 595-600.
3. *Jones J. G.* The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures // Freshwater Biology. 1977. V. 7. P. 67-91.
4. *Simon M., Tilzer M.* Bacterial response to seasonal changes in primary production and phytoplankton biomass in Lake Constance // J. Plankton Res. 1987. V. 9. P. 535-542.
5. *Relexans J. C., Meybeck M., Billen G., Brugeaille M., Etcheber H., Somville M.* Algal and microbial processes involved in particulate organic matter dynamics in the Loire estuary // Estuarine Goastal Shelf Sci. 1988. V. 27. P. 625-644.
6. *Sherr B. F., Sherr E. B., Pedros-Alio C.* Simultaneous measurement of bacterioplankton production and protozoan bacterivory in estuarine waters // Mar. Ecol. Prog. Ser. 1989. V. 54. P. 209-219.
7. *Healey M. J., Moll R. A., Diallo C. H.* Abundance and distribution of bacterioplankton in the Gambia River, West Africa // Microb. Ecol. 1988. V. 16. P. 291-310.
8. *Garnier J.* Bacterioplankton in the Seine River (France): impact of the Parisian urban effluent // Can. J. Microbiol. 1991. V. 38. P. 56-64.

9. *Holder-Franklin M. A., Thorpe A., Wuest L.* Evaluation of tests employed in the numerical taxonomy of river bacteria // J. Microbiol. Methods. 1992. V. 15. P. 263-277.

10. *Nakatsu C. H., Fulthorne R. R., Holland B. A., Peel M. C., Wyndham R. C.* The phylogenetic distribution of a transposable dioxygenase from the Niagara River watershed // Molecular Ecology. 1995. V. 4. P. 593-603.

11. *Glazebrook P. W., Moriarty D. J. W., Hayward A. C., MacRae I. C.* Seasonal changes in numbers and the location of a particular bacterial strain of *Alteromonas sp.* In seagrass sediments // Microb. Ecol. 1996. V. 31. P. 1-13.

12. *Garland J. L., Mills A. L.* Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization // Appl. Environ. Microbiol. 1991. V. 57. P. 2351-2359.

13. *Kettunen R. H., Rintala J. A.* Sequential anaerobic-aerobic treatments of sulphur rich phenolic leachates // J. Chem. Tech. Biotechnol. 1995. V. 62. P. 1-8.

14. *Hobbie J. E., Daley R. J., Jasoer S.* Use of Millipore filters for counting bacteria by fluorescence microscopy // Appl. Environ. Microbiol. 1977. V. 33. P. 1225-1228.

15. *Gibbs R. A., Hayes C. R.* The use of R2A medium and the spread plate method for the enumeration of heterotrophic bacteria in drinking water // Letters in Applied Microbiol. 1988. V. 6. P. 19-22.

16. *Smith G. B., Tiedje J. M.* Isolation and characterization of a nitrite reductase gene and its use as a probe for denitrifying bacteria // Appl. Environ. Microbiol. 1992. V. 58. P. 376-384.

17. Manual of practical works for microbiology (Jegorova N.S. ed.) Moscow University. 1983. P. 200-201. (in Russian).

18. *Adams M. H.* Bacteriophages. Interscience Publishers Inc. New-York. 1959. P. 445-447.

19. *Bauchop T., Elsden S. R.* The growth of microorganisms in relation to their energy supply // J. Gen. Microbiol. 1960. V. 23. P. 469-495.

20. *Leahy J. G., Colwell R. R.* Microbial degradation of hydrocarbons in the environment // Microbiological Reviews. 1990. V. 54. P. 305-315.

21. *Van der Meer J. R., de Vos W. M., Harayama S., Zehnder A. J. B.* Molecular mechanisms of genetic adaptation to xenobiotics compounds // Microbiological Reviews. 1992. V. 56. P. 677-694.

22. *Heinaru E., Talpsep E., Linnas A., Stottmeister U., Heinaru A.* Metabolic and genetic diversity of phenol-utilizing bacteria as an enhancer of natural biodegradation in polluted waters // Oil Shale. 1997. V. 14, No. 4 Special. P. 454-468.

23. *Körner H., Zumft W. G.* Expression of denitrification enzymes in response to the dissolved oxygen level and respiratory substrate in continuos culture of Pseudomonas stutzeri // Appl. Environ. Microbiol. 1989. V. 55. P. 1670-1676.

24. *Robertson L. A., Keunen J. G.* Aerobic denitrification: a controversy revived // Arch. Microbiol. 1984. V. 39. P. 351-354.
25. *Ward B. B.* Nitrification and denitrification: probing the nitrogen cycle in aquatic environments // Microb. Ecol. 1996. V. 32. P. 247-261.
26. *Hansson G.-B., Klemedtsson L., Stenström J., Torstensson L.* Testing in the influence of chemicals and soil autotrophic ammonium oxidation // Environmental Technology and Water Quality. 1991. B. 6. P. 351-360.
27. *Wünsche L., Brüggemann L., Babel W.* Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution // FEMS Microbiol. Ecol. 1995. V. 17. P. 295-306.

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