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EXECUTIVE SUMMARY

The complexity of environmental pollution, now reported all over the world, increases the demand for novel, highly efficient and cost-effective rehabilitation technologies. Biotechnologies are often regarded as attractive alternatives to other clean up technologies. Bioremediation and phytoremediation exploit the ability of certain microorganisms and plants to clean up contaminated sites, by either degradation, stabilization or accumulation processes. These technologies are gaining general acceptance as relatively low-cost potential alternatives to standard practices.

In this context, a common research effort has merged scientists from the United States and Poland, in the framework of a cooperative research initiative aiming to investigate and deploy bioremediation and phytoremediation for the clean up of contaminated sites in both countries. The present project, sponsored by and conducted for the United States Department of Energy (DOE), was undertaken by the Institute for Ecology of Industrial Areas (Katowice, Poland) in cooperation with Savannah River Technology Center and Florida State University.

This report describes the five tasks conducted in FY00. Two distinct types of pollutants were considered: organic contaminants including petroleum hydrocarbons and chlorinated solvents, as well as inorganic contaminants including metals such as lead and mercury. Because of the difference in chemical structure and properties of these contaminants, distinct approaches have been used for environmental remediation in each case. Consequently, the first three tasks focus on bioremediation of environments contaminated with petroleum hydrocarbons (task 1 and 2) or chlorinated solvents (task 3), while the two remaining tasks focus on phytoremediation of soils contaminated with lead (task 4) or mercury (task 5). The following is a brief description of each task.

Task 1 – Microbial Characterization The aim of this work was the complex characterization of a highly efficient hydrocarbon-degrading microbial community, previously isolated from petroleum contaminated slurry lagoons at Czechowice Oil Refinery, Poland. Isolated microbial strains, exclusively represented by yeasts and filamentous fungi, are able to degrade both chain and aromatic hydrocarbons (up to 95 %) at low pH values (< 2.5). This hydrocarbon degrading ability is pH and temperature dependent (with best results at pH 2.5 and at 29^0C). Each microbial strain was characterized thoroughly for morphology, enzyme production, substrate specificity, biomass and fatty acids production. Direct correlation between hydrocarbon degrading ability and production of biomass and membrane fatty acids

was observed for some of the studied strains. Other microbial communities (keratinolytic) were reported along with the acidophilic fungal community described above, including some rare species of scientific significance. This microbial community could provide the biological material for remediation of similar petroleum contaminated sites worldwide.

Task 2 – Bioreactor Project This project task determined the removal efficiency of organic contaminants from petroleum contaminated soils, using a mobile bioreactor. The work focused on the design, construction, start-up and operation of a continuous airflow, packed bed reactor. The system consists of the following: a standard container for waste collection and transport (roll off); supplementary equipment to control air flow, nutrient availability, moisture level, and leachate recycling; as well as a monitoring system allowing continuous process control. In the reactor, the bioventing process was used for remediation of petroleum contaminated soils from the Czechowice Oil Refinery area which were amended first with wood chips and fertilizers. Good degradation percentages (of approximately 50 % after 97 days) indicate the potential of the designed bioreactor system for the remediation of petroleum contaminated soils. Based on the present experiment, a series of improvements are proposed to the construction and operation of the bioreactor, monitoring system and sensors. In addition, the bioreactor will be operated to accept both contaminated water and soil, providing a useful tool for bioremediation of petroleum contaminated environments.

Task 3 – Bioremediation Project This task reviewed literature data concerning biodegradation of chlorinated solvents, focusing on the identification of factors and conditions that have been shown to enhance or inhibit the process. Experimental work also was carried out to initiate feasibility studies and biodegradation tests. The goal of the experimental activities was to conduct chemical and microbiological characterization of soil and groundwater from a metallurgical site in central Poland. The final goal was the preparation of a proposal to apply existing bioremediation expertise to solve critical problems posed by chlorinated solvents. Bioremediation of chlorinated solvents involves microorganisms that naturally are present in the subsurface and are able to degrade these solvents to environmentally acceptable products (i.e., carbon dioxide, chloride and water). The degradation process is usually anaerobic, but aerobic degradation also was reported for TCE. A combination of anaerobic and aerobic processes may be necessary to completely degrade the compounds. Addition of electron donors and nutrients alone stimulate the conversion of chlorinated solvents to intermediate toxic compounds. Additional steps will be needed for complete mineralization. Several field demonstrations have been performed using accelerated

bioremediation with the addition of benzoate, lactate, and/or methanol to stimulate the reductive dechlorination of PCE and TCE in groundwater. These experiments investigated the potential for and rates of biodegradation of TCE and its daughter compounds in soil under anaerobic conditions using anaerobic mixed-bacterial consortia obtained from wastewater treatment sludge. Results showed that under anaerobic conditions, TCE is biodegraded to isomeric DCE and vinyl chloride. Addition of sewage sludge to soil enhanced biodegradation of TCE and improved culture conditions. These results confirm the possibility of bioremediating soils contaminated with chlorinated solvents. Based on the present project, a work plan is proposed to develop a technology for bioremediation of chlorinated solvents in a bioreactor. A hybrid anaerobic and aerobic biodegradation mechanism supplemented with methanol, as an electron donor, will be considered.

Task 4 – Phytoremediation Project (Cost – Effective Phytoremediation) The aim of this task was to develop advanced technologies for amendment application to contaminated soils. This will result in reduction of material and application costs and decreased crop damage. For this purpose, a field-deployed soil amendment dispenser based on standard agricultural spray equipment was designed, built and tested. The dispenser was designed to apply amendments to the soil close to plant stems and to move easily through the field without damaging plants. The interaction between the degree of soil pollution and intensity of amendment application also was considered. In order to control amendment application, a computerized system was developed. This system uses site characterization data to determine the need for amendment application at any given location within the target site. A lead concentration of 300 mg/kg in soil was considered as the critical value for determining whether or not to apply amendments. Field tests were conducted on soil contaminated with mercury and cadmium that were planted with Indian mustard (*Brasica juncea*). Applying amendments in relation to soil lead concentrations resulted in overall savings of 35 % of the total phytoremediation cost. The current generation dispenser system combination of the dispenser + controller systems is now ready to be used in a full-scale deployment application.

Task 5 – Phytoremediation Project (Evaluation of Novel Mercury Remediation Technology) The purpose of this task was to identify and evaluate promising technologies for the remediation of mercury-contaminated soils. This information could be applied to treat mercury contamination at DOE sites such as Oak Ridge National Laboratory. An intensive literature review was carried out on current soil treatment technologies and their applicability. Soil contamination at a polluted site in Poland was characterized and several remediation

approaches were evaluated. The technologies considered included bioremediation and phytoremediation in addition to classic remediation techniques such as: chemical stabilization, thermal treatment, chemical extraction, soil washing, and electrochemical remediation. A chemical plant in southern Poland was chosen as the site for experimental work. The facility is a chemical manufacturing business that has been operating for over 70 years. Four mercury-contaminated sites were identified and characterized thoroughly for mercury content in soil at several depths. Results showed the greatest mercury concentrations in surface soils, but a downward migration of mercury also is of concern. Investigations were focused on the selection of chemical substances and plants capable to effectively bind/stabilize mercury and its compounds in soil. Microbial communities associated with different plant species were evaluated for their capacity in Hg remediation. Growth, yield, health and vitality of the investigated plant species were reported, as well as Hg distribution in plant parts. Results indicate that plants are able to grow in mercury contaminated soils at comparable rates to those grown in normal soil. Plants growing in Hg contaminated soil accumulate Hg mainly in the roots. Of the plants studied, willow accumulated the highest amount of mercury, but grass species (meadow grass and fescue) created a better soil penetration and stabilization system. Grass species will be considered for further applications. Lab-scale experiments were carried out for the selection of chemical substances to stabilize/bind mercury. Inexpensive chemical substances tested (i.e., fine and granular sulfur, zeolites or mixtures of dolomite and zeolite) are known for their ability to bind metals. All of the chemical substances added to the soil stabilized water soluble and exchangeable forms of mercury after 6 weeks. The chemical treatment can be used as either initial (interim) or final (polishing) treatment at mercury-contaminated sites.

Project Deliverable

Microbial Characterization

FY00 Final Report – Task 1

Molecular Characterization of Acidophilic Microorganisms Capable of Degrading Petroleum Hydrocarbons

Submitted to:
U.S. Department of Energy
National Energy Technology Laboratory
Morgantown, West Virginia

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In a previous project (Worsztynowicz et al., 1999), an acidophilic microbial community has been recovered from the petroleum waste-contaminated soils and waters at the Czechowice Oil Refinery (CZOR). This community consists of filamentous fungi, yeasts and bacteria that are able to degrade both chain and aromatic (PAHs) hydrocarbons at extremely low pH (2.5 or even lower). The combination of physical and chemical properties (temperature changes, low pH, and limited nutrient availability) of the refinery's environment has served as selective pressure factors directing the evolution of this community. Since, the microorganisms from the community had not been thoroughly characterized from molecular, physiological, and taxonomic points of view, this was the aim of this project. The project consisted of the following tasks:

- Isolation and purification of petroleum hydrocarbon-degrading and acidophilic strains;
- Examination of petroleum hydrocarbon-degrading strains for growth at pH 2.5;
- Morphological characterization of petroleum hydrocarbon-degrading and acidophilic fungal strains;
- Examination of the influence of temperature and pH on growth of acidophilic fungal strains;
- Characterization of petroleum hydrocarbon-degrading and acidophilic fungal strains for production of enzymes with the API ZYM bioMèrieux test;
- Identification of substrate specificity and spectrum for acidophilic fungal strains;
- Screening of acidophilic fungal strains for petroleum hydrocarbon removal rates and biomass production;
- Identification of yeasts with the API 20C AUX bioMèrieux system and with extended methods by CBS;
- Identification of fatty acids methyl esters (FAME);
- Additional characterization of the fungal community from the lagoons at the refinery;
- Conclusions;
- Summary.

Isolation and purification of petroleum hydrocarbon-degrading and acidophilic strains

In a previous project (Worsztynowicz *et al.*, 1999), microorganisms capable of degrading petroleum hydrocarbons were isolated from the clayey soil and the leachate from the biopile at the refinery and from the columns in a laboratory experiment. Four methods were used for isolation of these microorganisms from the above-mentioned materials. These were namely:

- Dilution method in phosphate buffer;
- Most Probable Number (MPN) method combined with the petroleum and naphthalene enrichment method with incubation in Biolog boxes;
- Enrichment and sequential enrichment methods in mineral medium (MM) supplemented with sterilized petroleum, naphthalene or other hydrocarbons in Erlenmeyer flasks; and
- Hydrocarbon(s) baiting method.

The dilution and MPN methods were those of U.S. EPA (1978). Schlegel (1996) has described the enrichment and sequential enrichment methods. The liquid mineral medium (MM) consisted of components A and B. The compositions of these components were the following:

Component A

NH_4NO_3 – 1 g
$MgSO_4*7H_2O$ – 0.2 g
$CaCl_2*H_2O$ – 0.03 g
Microelements solution – 1 mL
Distilled H_2O – 900 mL

Component B

KH_2PO_4 – 1 g
K_2HPO_4 – 1 g
Distilled H_2O – 100 mL

The composition of microelements was that of Schlegel (1996). The pH of the medium was adjusted to 6.8. After autoclaving, cooling down to the room temperature and mixing the components A and B, the medium was supplemented with sterilized hydrocarbon(s) (0.1% w/w), and soil or leachate (1-2% w/w). After four weeks of incubation in a rotary shaker at room temperature, microorganisms capable of degrading hydrocarbons were isolated using the 1:10 SMA medium supplemented with vitamins and incubated in oil and/or naphthalene vapors. The hydrocarbon(s) baiting method was similar to the hair baiting method (Vanbreuseghem, 1952) but, instead of hair, soil samples were covered with sterilized oil or solid/liquid hydrocarbons. The traditional Vanbreuseghem's hair baiting method was also used in this study. Altogether,

more than 150 strains of filamentous fungi, yeasts and bacteria were isolated and purified. Filamentous fungi and yeasts predominated in the collection of strains.

In this project, ten samples of sludge and soil were collected around the two sludge lagoons at the Czechowice Oil Refinery (third lagoon is now working as the biopile). Methods used for isolation of petroleum-degrading microorganisms were similar to those described above. However, the pH of the MM medium was adjusted to 2.5. After four weeks of incubation in a rotary shaker at room temperature, acidophilic strains were isolated using the Gelrite Gellan Gum (GGG) medium. The GGG medium consisted of components A and B. The compositions of these components were as follows:

Component A

Nutrient broth – 8 g
Yeast extract – 2.5 g
NaCl – 2.5 g
Distilled H_2O – 500 mL

Component B

Gelrite Gellan Gum (Sigma) – 20 g
Distilled H_2O – 500 mL

The pH of the component A was adjusted to 2.5 with 1M H_2SO_4. After autoclaving the components were cooled down to ca. 60^{o}C, mixed together and instantly poured into sterile Petri dishes. Isolated strains were purified and maintained also on GGG medium in the atmosphere of oil or naphthalene vapors.

Altogether, more than 50 acidophilic and petroleum-degrading strains were isolated and purified. All the strains were filamentous fungi and yeasts. Of these strains, 19 representative isolates were selected for further examination.

Examination of petroleum hydrocarbon-degrading strains for growth at pH 2.5

All (old and new) petroleum hydrocarbon-degrading strains were examined for growth at pH 2.5. These strains were transferred from maintaining GGG or 1:10 SMA + vitamins dishes or slants to 100 mL Erlenmeyer flasks containing 50 mL of pH 2.5 nutrient broth each and incubated for up

to 2 weeks. The turbidity, or the presence of mycelium, in the medium along with good growth on GGG medium after passages testified for acidophilic or acid tolerant nature of these strains.

Altogether 21 representative fungal strains capable of degrading petroleum hydrocarbons at pH 2.5 were selected for further examination. The strains were coded RF1-19 (19 new isolates) and R11-12 (2 old isolates).

Morphological characterization of petroleum hydrocarbon-degrading and acidophilic fungal strains

Subsequently, the petroleum hydrocarbon-degrading and acidophilic strains were identified morphologically. Both macro- and micromorphological characteristics of these strains were examined. Macromorphological examination included the features of colonies whereas micromorphological examination included microscopic features of the strains. Fungal strains were examined using the methods recommended by de Hoog & Guarro (1995). The strains were first examined on direct slides. Then, they were incubated on identification media, e.g., on MEA, CYA and PYE, and on media stimulating sexual reproduction, viz. Takashio agar (TK), rice (RA) and water (WA) agars. In order to observe the sporulating bodies of fungi, a microslide technique was employed.

Six isolates of yeasts and yeast-like fungi (RF1, RF3, RF4, RF5, RF6, and RF8) were shipped to the Centraalbureau voor Schimmelcultures (CBS) at Baarn (The Netherlands) for extended examination. Final results of fungal identification are presented in Table 1. Ten strains were filamentous fungi and eleven strains were classified as yeasts. Among the yeasts, *Candida saitoana* (six isolates) along with *Rhodotorula glutinis* (three isolates) were the predominating species. Among the filamentous fungi, *Pseudozyma* sp. (four isolates) and *Penicillium* spp. (three isolates) were most common in the collection.

Table 1. Results of preliminary identification of the examined fungal strains

Strain no.	Fungal group	Species
RF1	yeast	*Candida saitoana*
RF2	Filamentous fungus	*Arthrographis* sp.
RF3	Filamentous fungus	*Exophiala* sp.
RF4	yeast	*Rhodotorula glutinis*
RF5	yeast	*Candida saitoana*
RF6	Filamentous fungus	*Pseudozyma* sp.
RF7	yeast	*Candida saitoana*
RF8	Filamentous fungus	*Hyphopichia burtonii*
RF9	Filamentous fungus	*Penicillium* sp. I
RF10	yeast	*Candida saitoana*
RF11	filamentous fungus	*Pseudozyma* sp.
RF12	filamentous fungus	*Penicillium* sp. I
RF13	yeast	*Rhodotorula* sp.
RF14	yeast	*Rhodotorula glutinis*
RF15	filamentous fungus	*Pseudozyma* sp.
RF16	yeast	*Rhodotorula* sp.
RF17	filamentous fungus	*Penicillium* sp. II
RF18	yeast	*Rhodotorula glutinis*
RF19	filamentous fungus	*Pseudozyma* sp.
R11	yeast	*Candida saitoana*
R12	yeast	*Candida saitoana*

Examination of the influence of temperature and pH on growth of acidophilic fungal strains

The petroleum hydrocarbon-degrading and acidophilic fungal strains were tested for growth at different temperatures and pH. GGG medium was used for the tests. In the pH test, the pH of this medium was adjusted to 2.5, 5.0, 6.0, 7.0 and 8.0. Petri dishes were inoculated with 5 μL of homogenous suspension of propagules of a given strain and incubated in the dark at room temperature. After 2, 4, 8 and 10 days of incubation, the diameters of colonies were measured. In the temperature test, inoculated Petri dishes containing the pH 2.5 GGG medium were incubated at 20, 29, 37 and 45°C. After 2, 4, 8 and 10 days of incubation in the dark, the diameters of colonies were measured. Daily growth rates were calculated for comparison of fungal growth on GGG media at different temperatures and pH.

Daily growth rates at different pH are presented in Table 2. In respect to pH, the whole collection of fungal acidophilic strains is characterized in Figure 1. In general, the highest mean

daily growth rate was observed at pH 2.5. The means for daily growth rates decreased with increasing pH. However, these differences were not statistically significant (ANOVA test). The strains RF2, RF3, RF6, RF7, RF8, RF9, RF12, and RF15 had the highest daily growth rates at pH 2.5 and belonged to many species (*Arthrographis* sp., *Exophiala* sp., *Pseudozyma* sp., *Candida saitoana*, *Hyphopichia burtonii*, and *Penicillium* sp. I). Most of the strains examined were able to grow even at pH 2.5.

Table 2. Daily growth rates (mm/day) of fungal strains at different pH

Strain no.	**Daily growth rate (mm/day) at**				
	pH 2.5	**pH 5**	**pH 6**	**pH 7**	**pH 8**
RF1	0.75	0.50	0.50	0.77	0.45
RF2	2.38	2.50	1.85	1.07	0.00
RF3	4.25	2.82	1.93	1.53	0.05
RF4	0.38	0.60	0.65	0.62	0.45
RF5	0.25	0.40	0.47	0.42	0.30
RF6	1.63	0.80	0.83	0.92	0.87
RF7	0.63	0.42	0.37	0.30	0.20
RF8	2.38	1.17	0.83	0.62	0.10
RF9	6.13	4.62	3.85	3.88	2.92
RF10	0.40	0.45	0.45	0.50	0.33
RF11	1.13	0.73	1.03	0.93	0.85
RF12	7.50	4.90	3.67	2.65	1.40
RF13	0.23	0.53	0.55	0.38	0.47
RF14	0.50	0.47	0.47	0.60	0.43
RF15	1.50	1.08	0.88	0.80	0.32
RF16	0.25	0.33	0.40	0.38	0.42
RF17	4.00	5.35	4.98	4.35	3.27
RF18	0.50	0.43	0.37	0.43	0.38
RF19	0.50	0.75	0.78	0.75	0.75
R11	0.37	0.45	0.37	0.50	0.40
R12	0.25	0.32	0.55	0.65	0.40

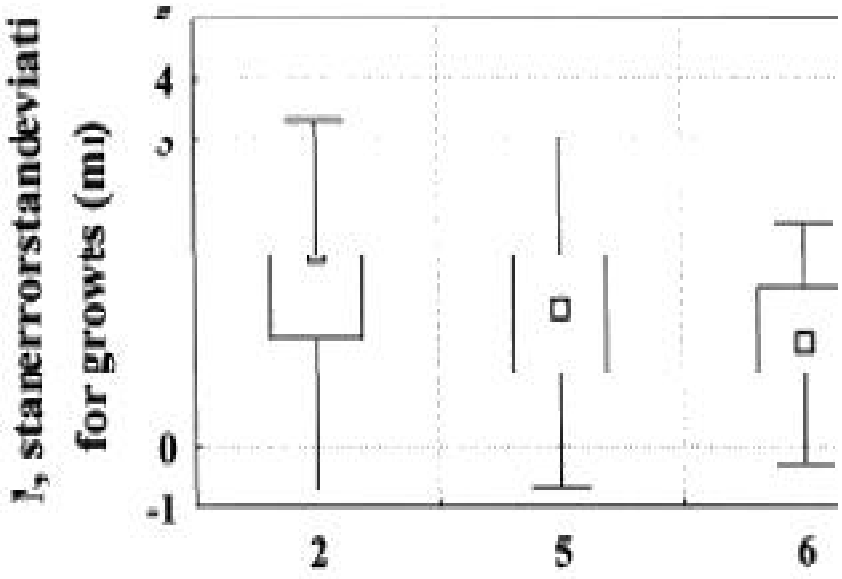


Figure 1. Characterization of the whole fungal collection in relation to pH

Daily growth rates at different temperatures are shown in Table 3. In respect to temperature, the whole collection of fungal acidophilic strains is characterized in Figure 2. Generally, the highest mean daily growth rate was noticed at 29° C. Only some strains were able to grow at 45° C. Except for strain RF3 (*Exophiala* sp., with a relatively good growth observed), however, most strains showed scant growth at that high temperature. Differences in daily growth rates at different temperatures were statistically significant (ANOVA test).

Table 3. Fungal daily growth rates at different temperatures

Strain no.	Daily growth rates at			
	20°C	29°C	37°C	45°C
RF1	0.88	0.92	0.58	0.00
RF2	3.17	3.25	2.25	0.67
RF3	5.12	6.08	3.05	2.00
RF4	0.57	0.77	0.63	0.13
RF5	0.72	0.83	0.62	0.00
RF6	1.24	1.55	0.57	0.00
RF7	0.82	0.93	0.80	0.10
RF8	2.35	3.40	3.32	0.00
RF9	6.22	9.32	7.41	0.00
RF10	0.73	0.93	0.78	0.02
RF11	3.55	3.08	1.77	0.00
RF12	8.49	10.13	0.77	0.00
RF13	0.53	0.70	0.60	0.00
RF14	0.57	0.80	0.68	0.07
RF15	1.75	2.22	0.62	0.00
RF16	0.48	0.78	0.47	0.15
RF17	6.67	8.75	4.85	0.00
RF18	0.63	0.73	0.83	0.13
RF19	1.20	1.33	0.75	0.00
R11	0.80	0.75	0.73	0.00
R12	0.60	0.97	0.73	0.00

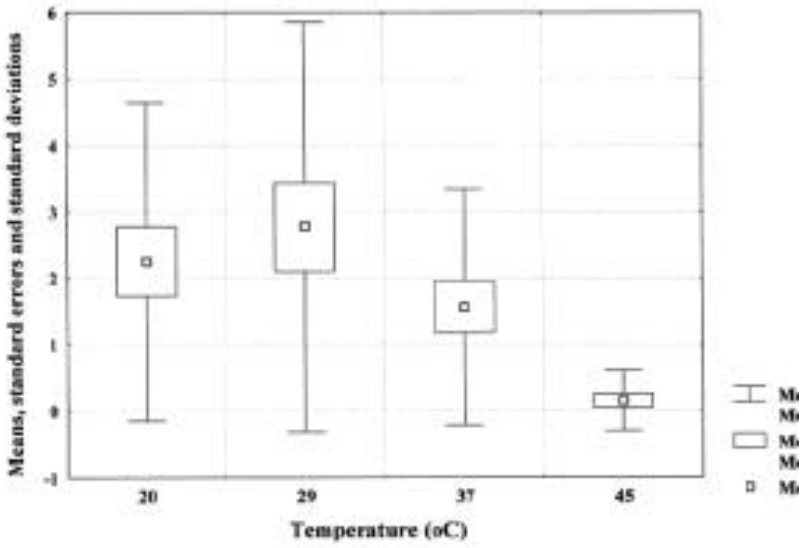


Figure 2. Characterization of the whole fungal collection in respect to temperature

Characterization of petroleum hydrocarbon-degrading and acidophilic fungal strains for production of enzymes with the API ZYM bioMèrieux test

Petroleum hydrocarbon-degrading and acidophilic fungal strains were tested for their ability to produce different enzymes. The first part of this task employed the API ZYM bioMèrieux system while the second part used traditional methods with detection of enzyme production on solid media (see next chapter).

The API ZYM bioMèrieux system is a semiquantitative technique that allows examination the production of 19 constitutive enzymes. A suspension of propagules of each strain was prepared with turbidity between MacFarland numbers 5 and 6 as standard. Pure cultures from slants were used to prepare the suspension. With a Pasteur pipette, 65 µL of specimen were inoculated in each capsule of the test strip. A plastic lid was placed on the trays following inoculation, and they were incubated for 4 hrs at 28°C. The inoculated strips were protected from bright light. After incubation, ampoules of the API ZYM reagents were opened and one drop of reagent A and one drop of reagent B were added. The color developed after 5 minutes. Negative reactions were colorless. The 19 enzymes assayed were alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, α-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, Naphthol-AS-BI-phsphohydrolase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The APIZYM reagent A consisted of Tris (hydroxymethyl) aminomethane (250 g), HCl (37%; 110 mL), lauryl sulphate (100 g) and distilled water (1000 mL). The reagent B consisted of Fast Blue BB (3.5 g) and 2-methoxyethanol to make 1000 mL.

The fungal strains examined differed in the qualitative and quantitative spectra for production of constitutive enzymes (Table 4). Certain enzymes such as esterase (C 4), esterase lipase (C 8), leucine arylamidase, valine arylamidase, phosphatase alkaline, and Naphthol-AS-BI-phosphatase were produced by all strains while other enzymes such as β-glucuronidase, and β-fucosidase were not detected in any strain. The strains RF 6, 9, 11, 12, and 19 (*Pseudozyma* sp. and *Penicillium* sp. I) produced the highest quantities of enzymatic activity products while the strain RF8 (*Hyphopichia burtonii*) showed the smallest enzyme production. In the strains of *Candida saitoana*, *Rhodotorula glutinis* and *Pseudozyma* sp., the homogeneity in the constitutive enzyme spectra was observed. In the *Candida saitoana* strains, only the strain RF11 showed a higher

activity of β-glucosidase. In the strains of *Rhodotorula*, the strains RF13 and RF16 (*Rhodotorula* sp.) displayed lower activities of β-glucosidase than the strains of *Rhodotorula glutinis*. By contrast, the enzyme production spectrum of the *Penicillium* strains was heterogeneous. The strain RF17 differed from the strains RF9 and RF12 in the lower activity of esterase lipase (C 8), valine arylamidase, α-galactosidase, and α-mannosidase. Also, the lower activity of α-galactosidase characterized the strain RF9.

Table 4. Constitutive enzymes produced by acidophilic and petroleum hydrocarbon-degrading fungal strains. Data obtained with the API ZYM bioMèrieux test

Enzymes	RF1	RF2	RF3	RF4	RF5	RF6	RF7	RF8	RF9	RF10	RF11	RF12	RF13	RF14	RF15	RF16	RF17	RF18	RF19	R11	R12
Phosphatase alcaline	0*	0-5	>40	20	0	>40	0-5	0-5	>40	0-1	20	>40	20	30	30	0-5	>40	30	>40	0	0-5
Esterase (C 4)	10	10	30	20	10	20	10	20	30	5	20	30	10	20	20	>40	20	30	10	20	10
Esterase Lipase (C 8)	20	10	30	30	20	30	20	10	20	>40	20	30	30	>40	10	>40	0-5	30	10	30	30
Lipase (C 14)	0	0	0	0	0	10	0-5	0	0-5	0	0-5	10	0	0	0-1	0	0	0	0	0	0
Leucine arylamidase	>40	>40	>40	>40	>40	>40	>40	20	>40	>40	>40	>40	>40	>40	30	>40	>40	>40	>40	>40	>40
Valine arylamidase	10	10	20	>40	30	20	30	10	20	40	30	20	>40	>40	20	>40	5	>40	20	>40	30
Cystine arylamidase	20	10	0	30	20	10	20	0-5	0-5	40	20	0	30	30	10	>40	5	30	10	>40	30
Trypsin	0-5	0-5	0	0-5	0-5	0-5	0-5	0	0-5	0-5	0-5	0	20	10	0-5	0	0	5	5	0-5	10
Chymotrypsin	0-5	0-5	0	0-5	0-5	0-5	0-5	0	0-5	0	0	0	0-5	0-5	0-5	0	0	0-5	0-5	0-5	0-5
Phosphatase acid	>40	>40	>40	>40	>40	>40	>40	10	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40	>40
Naphthol-AS-BI-phosphohydrolase	10	30	30	>40	10	>40	10	10	>40	0-5	>40	>40	>40	>40	>40	>40	>40	>40	>40	0-5	5
α-galactosidase	0	0	0	0	0	30	0	0	30	0	30	20	0	0	30	5	0	0	>40	0	0-5
β-galactosidase	0	0	0	0	0	20	0-5	0	0-5	0	10	>40	0	0	20	5	20	0	30	0	0
β-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-glucosidase	>40	10	0	0-5	>40	30	>40	0-1	20	>40	30	>40	0-5	0-5	30	0	10	0-5	>40	>40	>40
β-glucosidase	0	>40	20	20	0-5	30	0-5	0-1	>40	0	>40	>40	0-5	30	20	0	>40	10	30	30	0
N-acetyl-β-glucosaminidase	>40	>40	>40	0	>40	30	>40	0-1	>40	>40	>40	>40	0	0	30	0	>40	0-5	30	>40	>40
α-mannosidase	0	0	0	0	0	30	0	0	10	0	30	>40	0	0	20	0	0	0	30	0	0
α-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* - amounts of products of enzymatic reactions in nanomoles

Identification of substrate specificity and spectrum for acidophilic fungal strains

Amylase production was tested on Bacto nutrient agar (Difco) containing 0.2% soluble starch (Hankin & Anagnostakis, 1975). After incubation Petri dishes were flooded with an iodine solution and a yellow halo around the colonies in an otherwise blue medium indicated the enzyme production. Cellulolytic activity was tested on Bravery's mineral salt medium (1968) supplemented with carboxymethylcellulose for the production of CMCase. Strains were incubated at 20°C for 20 days and checked every second day for the appearance of clear halos in the opaque medium. Deoxyribonuclease activity was detected on DNAse test agar (Difco). After incubation Petri dishes were flooded with 1M HCl. DNA depolymerization was observed by clear zones surrounding the colonies. Lipolysis was assessed on Sierra's medium (1957) with Tween 80 as substrate. Lipolytic activity was indicated by the appearance of deposits of calcium salts formed by liberated fatty acids. The polygalacturonase production test followed the method of Vaughn *et al.* (1957) with sodium polypectate as a substrate. Gel softening around the colonies indicated the enzyme production (Hankin *et al.*, 1971). After incubation Petri dishes were flooded with a 1% aqueous solution of hexadecylmetrimethylammonium bromide and clear zones around the colonies showed pectin degradation in an otherwise opaque medium. Phosphatase activity was tested on SMA agar (Difco) supplemented with 2% phenolphthalein diphosphate (sodium salt) as described by Hankin & Anagnostakis (1975). After incubation Petri dishes were opened and inverted over a container with ammonium hydroxide. Colonies of phosphatase-producing strains turned pink to red. Gelatin liquefaction was detected on Van der Walts' medium (1970). Petri dishes were incubated at 20°C for 10-12 days and proteolysis was revealed by medium liquefaction. Urease production was tested on Christensen's urea agar (Seeliger, 1956) and urea hydrolysis was indicated by the appearance of a deep pink color during incubation. The method by Hankin & Anagnostakis (1975) was intended to be used for examination of chitinase activity. Due to the high cost of pure chitin, however, the assessment of chitinase activity was abandoned. The method for catalase detection was that of U.S. EPA (1978).

The results concerning the production of selected enzymes on solid media are presented in Table 5. All strains produced catalase and phosphatase. No protease, DNA-ase, CMC-ase, pectinase and polygalacturonase activities were noticed. The majority of the strains examined produced lipase, amylase and urease. The highest number of enzymes (five) was produced by the strains RF2 (*Arthrographis* sp.), RF3 (*Exophiala* sp.), RF4, RF14, RF18 (*Rhodotorula glutinis*), RF17

(*Penicillium* sp. II) and RF19 (*Pseudozyma* sp.). The lowest number of enzymatic activities (two) was observed in all strains of *Candida saitoana* (RF1, RF5, RF7, RF10, RF11, and RF12).

Table 5. Production of selected enzymes by acidophilic fungal strains.
Data obtained on solid media

Strain no.	Amylase	DNA-ase	Lipase	Catalase	Phosphatase	Urease	Pectinase	Polygalacturonase	Protease	CMC-ase
RF1	-	-	-	+	+	-	-	-	-	-
RF2	+	-	+	+	+	+	-	-	-	-
RF3	+	-	+	+	+	-	-	-	-	-
RF4	+	-	+	+	+	+	-	-	-	-
RF5	-	-	-	+	+	-	-	-	-	-
RF6	-	-	+	+	+	+	-	-	-	-
RF7	-	-	-	+	+	-	-	-	-	-
RF8	-	-	+	+	+	-	-	-	-	-
RF9	+	-	-	+	+	+	-	-	-	-
RF10	-	-	-	+	+	-	-	-	-	-
RF11	-	-	+	+	+	+	-	-	-	-
RF12	-	-	+	+	+	-	-	-	-	-
RF13	-	-	+	+	+	+	-	-	-	-
RF14	+	-	+	+	+	+	-	-	-	-
RF15	-	-	+	+	+	+	-	-	-	-
RF16	-	-	+	+	+	+	-	-	-	-
RF17	+	-	+	+	+	+	-	-	-	-
RF18	+	-	+	+	+	+	-	-	-	-
RF19	+	-	+	+	+	+	-	-	-	-
R11	-	-	-	+	+	-	-	-	-	-
R12	-	-	-	+	+	-	-	-	-	-

+ - enzyme production detected
- - no enzyme production

Screening of strains for petroleum hydrocarbon removal rates and biomass production

Initially, testing of petroleum hydrocarbon-degrading and acidophilic fungal strains for hydrophobicity and hydrophilic activity was intended. After a preliminary experiment, however, it was felt that the above test was highly inaccurate, particularly when filamentous fungi were under examination. Instead of this test, a hydrocarbon removal and biomass production experiment including all selected acidophilic fungal strains from the refinery was performed. The experiment determined the petroleum hydrocarbon removal and biomass production rates for the strains. Initially, determination of hexadecane, pristane or undecane removal rates in the pH 2.5 liquid mineral medium (MM) inoculated with the strains was considered. However, after a

detail study of our previous results and also literature data it was decided to use autoclaved petroleum from the refinery as the only carbon source for fungi in this experiment. As a mixture of many high-weight chain (branched and non-branched) hydrocarbons, the autoclaved petroleum is much better available to microorganisms than pure hydrocarbons. The petroleum hydrocarbon removal rates were determined with a GC/MS device in isooctane extracts. Biomass production was measured with a gravimetric method.

The biomass production values ranged between 0.0-17.44 mg per sample while the petroleum hydrocarbon removal rates fluctuated between 42.98-94.63% (0.24-0.80 mg/day/sample) after three weeks of incubation (Table 6). Figure 3 displays the correlation between petroleum hydrocarbon removal rates and biomass production by the acidophilic fungal strains. The correlation coefficient R was rather low. It can be seen, however, that three strains of *Penicillium* (RF 9, 12 and 17) and *Exophiala* sp. (RF 3) had the highest hydrocarbon removal rates and biomass productions while the lowest values of these parameters were observed in the strains RF16 (*Rhodotorula* sp.), RF14 and RF 18 (*Rhodotorula glutinis*).

Table 6. Petroleum hydrocarbon mass losses and biomass production by acidophilic fungal strains

Sample no.	Biomass (mg/sample)	Petroleum hydrocarbon mass loss (%)
RF1	0.31	75.25
RF2	6.63	68.81
RF3	10.42	88.29
RF4	1.66	76.04
RF5	0.77	66.34
RF6	0.00	76.75
RF7	0.13	76.72
RF8	0.38	82.45
RF9	15.57	91.71
RF10	2.06	63.41
RF11	5.24	77.10
RF12	11.62	94.63
RF13	8.77	64.33
RF14	4.56	42.98
RF15	2.94	68.53
RF16	0.53	46.21
RF17	17.44	93.66
RF18	1.44	48.42
RF19	1.94	67.79
R11	2.58	56.79
R12	0.67	58.34

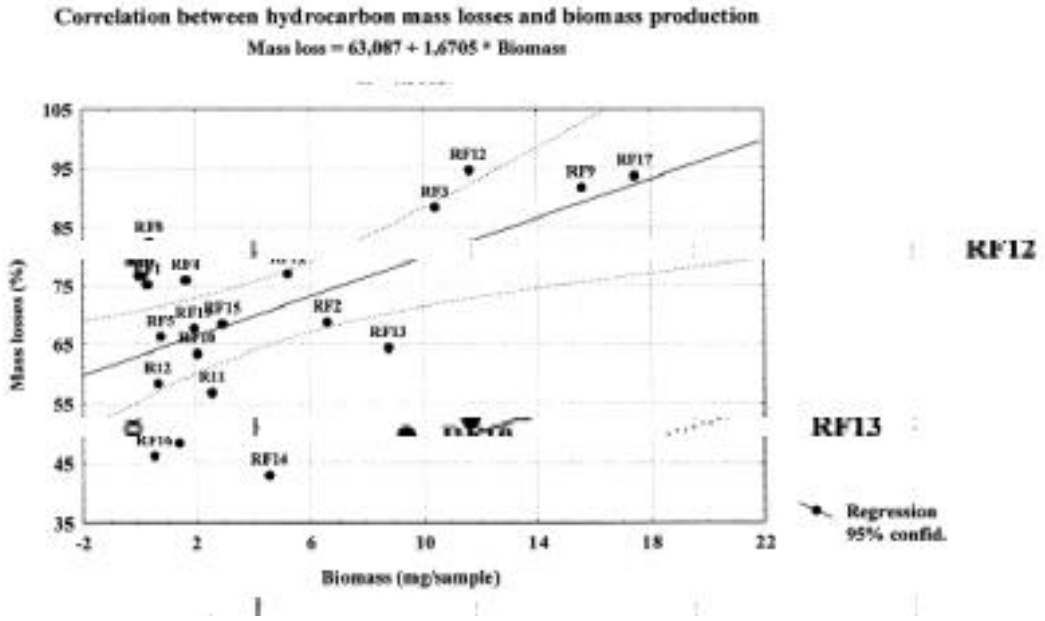


Figure 3. Correlation between biomass production and petroleum hydrocarbon mass losses for acidophilic fungal strains

Identification of yeasts with the API 20C AUX bioMèrieux system and with extended methods by CBS

The API 20C AUX bioMèrieux system is designed for the precise identification of the most frequently encountered yeasts in medical laboratories. The API 20C AUX strip consists of 20 microtubes containing dehydrated substrates in which 19 assimilation tests are performed. The capsules on the strip are inoculated with a minimal medium and the yeasts only grow if they are capable of utilizing each substrate as a sole carbon source. The presence of hyphae or pseudohyphae (observed on RAT medium) is considered as the 21st test of the system. The reactions are interpreted by comparison to controls and the identification is made using the Analytical Profile Index. In each case additional test, i.e., determination of microscopic and macroscopic features, is necessary to confirm the identification.

The yeasts identification results obtained with the API 20C AUX bioMèrieux system are presented in Table 7. In Table 8 the explanation of the abbreviations from Table 7 is given. As has been mentioned, the API 20C AUX bioMèrieux system is designed for identification of the most important yeasts of medical importance. Therefore, precise identification of all acidophilic yeasts from the refinery was not possible. As can be seen in Table 7, the identification of the yeasts at CBS differs from those obtained with the API 20C AUX bioMèrieux system. The CBS

results are shown in tables 9-11. The CBS results only confirmed the identification of *Rhodotorula glutinis*. At CBS all white yeasts were determined as *Candida saitoana*. Two pink *Rhodotorula* sp. strains were not shipped to CBS. These strains differ from *Rhodotorula glutinis* in the growth on cellobiose and lactose.

Identification of substrate specificity and spectrum for acidophilic fungal strains

Amylase production was tested on Bacto nutrient agar (Difco) containing 0.2% soluble starch (Hankin & Anagnostakis, 1975). After incubation Petri dishes were flooded with an iodine solution and a yellow halo around the colonies in an otherwise blue medium indicated the enzyme production. Cellulolytic activity was tested on Bravery's mineral salt medium (1968) supplemented with carboxymethylcellulose for the production of CMCase. Strains were incubated at 20°C for 20 days and checked every second day for the appearance of clear halos in the opaque medium. Deoxyribonuclease activity was detected on DNAse test agar (Difco). After incubation Petri dishes were flooded with 1M HCl. DNA depolymerization was observed by clear zones surrounding the colonies. Lipolysis was assessed on Sierra's medium (1957) with Tween 80 as substrate. Lipolytic activity was indicated by the appearance of deposits of calcium salts formed by liberated fatty acids. The polygalacturonase production test followed the method of Vaughn *et al.* (1957) with sodium polypectate as a substrate. Gel softening around the colonies indicated the enzyme production (Hankin *et al.*, 1971). After incubation Petri dishes were flooded with a 1% aqueous solution of hexadecylmetrimethylammonium bromide and clear zones around the colonies showed pectin degradation in an otherwise opaque medium. Phosphatase activity was tested on SMA agar (Difco) supplemented with 2% phenolphthalein diphosphate (sodium salt) as described by Hankin & Anagnostakis (1975). After incubation Petri dishes were opened and inverted over a container with ammonium hydroxide. Colonies of phosphatase-producing strains turned pink to red. Gelatin liquefaction was detected on Van der Walts' medium (1970). Petri dishes were incubated at 20°C for 10-12 days and proteolysis was revealed by medium liquefaction. Urease production was tested on Christensen's urea agar (Seeliger, 1956) and urea hydrolysis was indicated by the appearance of a deep pink color during incubation. The method by Hankin & Anagnostakis (1975) was intended to be used for examination of chitinase activity. Due to the high cost of pure chitin, however, the assessment of chitinase activity was abandoned. The method for catalase detection was that of U.S. EPA (1978).

The results concerning the production of selected enzymes on solid media are presented in Table 5. All strains produced catalase and phosphatase. No protease, DNA-ase, CMC-ase, pectinase and polygalacturonase activities were noticed. The majority of the strains examined produced lipase, amylase and urease. The highest number of enzymes (five) was produced by the strains RF2 (*Arthrographis* sp.), RF3 (*Exophiala* sp.), RF4, RF14, RF18 (*Rhodotorula glutinis*), RF17

Table 7. Results of identification of yeast strains with the API 20C AUX bioMèrieux system and the extended CBS examination

Strain no.	API 20C AUX results	Extended CBS examination	glu	gly	2kg	ara	xyl	Ado	xlt	gal	ino	sor	mdg	Nag	cel	lac	mal	sac	tre	mlz	raf
RF5	Candida famata	Candida saitoana	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
R11	Candida famata	Candida saitoana	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
R12	Candida famata	Candida saitoana	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
RF1	Unidentified	Candida saitoana	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
RF7	Unidentified	Candida saitoana	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
RF10	Unidentified	Candida saitoana	+	+	-	-	+	+	-	+	-	+	+	+	+	+	+	+	+	+	-
RF4	Rhodotorula glutinis	Rhodotorula glutinis	+	+	-	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+
RF14	Rhodotorula glutinis	Rhodotorula glutinis	+	+	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	+
RF18	R. glutinis/rubra	Rhodotorula glutinis	+	+	-	+	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+
RF13	Rhodotorula sp.	Not sent	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
RF16	Rhodotorula sp.	Not sent	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+

Table 8. Explanation of the abbreviations from Table 7

Abbreviation	Substrate
glu	Glucose
gly	Glycerol
2kg	2-keto-D-gluconate
ara	L-arabinose
xyl	D-xylose
ado	Adonitol
xlt	Xylitol
gal	Galactose
ino	Inositol
sor	Sorbitol
mdg	β-methyl-D-glucoside
nag	N-acetyl-D-glucosamine
cel	Celobiose
lac	Lactose
mal	Maltose
sac	Saccharose/sucrose
tre	Trehalose
mlz	Melezitose
raf	Rafinose

Table 9. The CBS characteristics of the strain RF1

Morphology:					
Pink colonies	-	Budding cells	+		
Lemon-shaped cells	-	Buds on stalks	-	Splitting cells	-
Filamentous	-	Pseudohyphae	-	Septate hyphae	-
Arthroconidia	-	Ballistoconidia	-	Symmetric ballistoconidia	-
Ascospores	-				
Fermentation:					
D-Glucose	-	Maltose	-	Lactose	-
D-Galactose	-	Sucrose	-	Raffinose	-
Growth on C compounds:					
D-Glucose	+	Maltose	+	Glycerol	+
D-Galactose	+	Trehalose	+	Erythriol	-
L-Sorbose	-	Methyl-D-glucoside	+	D-Glucitol	+
D-Glucosamine	+	Cellobiose	+	D-Mannitol	+
D-Ribose	-	Melibiose	-	myo-Inositol	-
D-Xylose	+	Lactose	+	2Keto-D-gluconate	-
L-Arabinose	-	Raffinose	+	D-Gluconate	+
L-Rhamnose	-	Melezitose	+	D-Glucuronate	-
Sucrose	+			DL-Lactate	-
				Butane 2,3-diol	+
Growth on N compounds:					
Nitrate	-	Ethylamine	+	L-Lysine	+
Cadaverine	+	D-glucosamine	-	D-tryptophan	-
Growth with:					
0,01% Cycloheximide	+	Acetic acid production	-		
Growth at: 37ºC	+	42ºC	-		

Determined as: Candida saitoana

Table 10. The CBS characteristics of the strain RF5

Morphology:					
Pink colonies	-	Budding cells	+		
Lemon-shaped cells	-	Buds on stalks	-	Splitting cells	-
Filamentous	-	Pseudohyphae	-	Septate hyphae	-
Arthroconidia	-	Ballistoconidia	-	Symmetric ballistoconidia	-
Ascopores	-				
Fermentation:					
D-Glucose	w	Maltose	-	Lactose	-
D-Galactose	-	Sucrose	-	Raffinose	-
Growth on C compounds:					
D-Glucose	+	Maltose	+	Glycerol	+
D-Galactose	+	Trehalose	+	Erythriol	-
L-Sorbose	-	Methyl-D-glucoside	+	D-Glucitol	+
D-Glucosamine	+	Cellobiose	+	D-Mannitol	+
D-Ribose	-	Melibiose	-	myo-Inositol	-
D-Xylose	+	Lactose	+	2Keto-D-gluconate	+
L-Arabinose	-	Raffinose	+	D-Gluconate	+
L-Rhamnose	-	Melezitose	+	D-Glucuronate	-
Sucrose	+			DL-Lactate	-
				Quinic acid	-
Growth on N compounds:					
Nitrate	-	Ethylamine	+	L-Lysine	+
Cadaverine	+	D-glucosamine	-	D-tryptophan	-
Growth with:					
0.01% Cycloheximide	+	Acetic acid production	-		
Growth at: 37ºC	+	40ºC	-		

Determined as: Candida saitoana

Table 11. The CBS characteristics of the strain RF4

Morphology:					
Pink colonies	+	Budding cells	+		
Lemon-shaped cells	-	Buds on stalks	-	Splitting cells	-
Filamentous	-	Pseudohyphae	-	Septate hyphae	-
Arthroconidia	-	Ballistoconidia	-	Symmetric ballistoconidia	-
Ascopores	-				
Fermentation:					
D-Glucose	-	Maltose	-	Lactose	-
D-Galactose	-	Sucrose	-	Raffinose	-
Growth on C compounds:					
D-Glucose	+	Maltose	+	Glycerol	+
D-Galactose	+	Trehalose	+	Erythriol	-
L-Sorbose	-	Methyl-D-glucoside	+	D-Glucitol	+
D-Glucosamine	-	Cellobiose	-	D-Mannitol	+
D-Ribose	+	Melibiose	-	myo-Inositol	-
D-Xylose	+	Lactose	-	2Keto-D-gluconate	-
L-Arabinose	+	Raffinose	+	D-Gluconate	-
L-Rhamnose	-	Melezitose	+	D-Glucuronate	-
Sucrose	+			DL-Lactate	-
Growth on N compounds:					
Nitrate	+	Ethylamine	+	L-Lysine	+
Cadaverine	+	D-glucosamine	-	D-tryptophan	+
Growth with:					
0,01% Cycloheximide	+	Acetic acid production	-		
Growth at: 37°C	+				

Determined as: Rhodotorula glutinis

Table 12. The mean contents and standard deviations for membrane (free) fatty acids measured as their methyl esters in four acidophilic fungal strains with the highest hydrocarbon removal efficiency

Strain no.	Statistics	Fatty acids content in biomass [%]	C8:0 [%]	C10:0 [%]	C12:0 [%]	C13:0 [%]	C14:1n9c [%]	C14:0 [%]	C15:0 [%]	C16:1n9c [%]	C16:0 [%]	C17:0 [%]	C18:1n9t + C18:1n9c + C18:2n6c [%]	C18:3n3 [%]	C18:0 [%]	C20:1 [%]	C20:0 [%]	C22:1n9 [%]	C22:0 [%]
RF2	Mean	0.66	0.32	0.28	0.53	0.14	0.01	1.21	0.14	1.18	20.11	1.29	65.66	4.01	3.65	0.54	0.35	0.28	0.30
	St. dev.	0.09	0.09	0.05	0.13	0.04	0.00	0.57	0.05	0.08	0.80	0.37	1.46	1.21	0.49	0.03	0.03	0.07	0.10
RF3	Mean	1.00	0.10	0.04	0.09	0.01	0.01	0.27	0.20	0.52	18.45	0.81	55.82	9.39	13.38	0.26	0.28	0.11	0.25
	St. dev.	0.32	0.01	0.01	0.01	0.00	0.00	0.03	0.03	0.02	0.29	0.13	1.73	1.40	0.91	0.05	0.04	0.04	0.02
RF9	Mean	1.34	0.09	0.02	0.04	0.01	0.01	0.28	0.44	0.98	19.68	0.93	66.39	2.51	7.74	0.31	0.32	0.05	0.20
	St. dev.	0.96	0.06	0.01	0.03	0.00	0.00	0.01	0.01	0.21	0.77	0.26	2.56	0.27	1.65	0.02	0.09	0.04	0.06
RF12	Mean	2.42	0.04	0.03	0.05	0.01	0.01	0.24	0.76	0.68	16.40	1.54	66.67	7.88	3.25	0.34	0.21	1.64	0.26
	St. dev.	0.54	0.00	0.01	0.01	0.00	0.00	0.08	0.09	0.12	0.97	0.08	1.19	0.97	0.45	0.02	0.07	1.60	0.06
RF17	Mean	0.46	0.10	0.10	0.15	0.01	0.01	0.33	0.57	0.54	21.77	1.13	63.38	2.01	5.52	1.11	0.61	2.00	0.68
	St. dev.	0.04	0.02	0.02	0.00	0.00	0.00	0.07	0.01	0.09	0.85	0.11	1.99	0.41	0.83	0.99	0.24	2.66	0.19

Abbreviations:
C8:0 = Caprylic Acid Methyl Ester
C10:0 = Capric Acid Methyl Ester
C12:0 = Lauric Acid Methyl Ester
C13:0 = Tridecanoic Acid Methyl Ester
C14:1n9c = Myristoleic Acid Methyl Ester
C14:0 = Myristic Acid Methyl Ester
C15:0 = Pentadecanoic Acid Methyl Ester
C16:1n9c = Palmitoleic Acid Methyl Ester
C16:0 = Palmitic Acid Methyl Ester
C17:0 = Heptadecanoic Acid Methyl Ester
C18:1n9t = Elaidic Acid Methyl Ester
C18:1n9c = Oleic Acid Methyl Ester
C18:2n6c = Linoleic Acid Methyl Ester
C18:3n3 = Linolenic Acid Methyl Ester
C18:0 = Stearic Acid Methyl Ester
C20:1 = cis-11-Eicosenoic Acid Methyl Ester
C20:0 = Arachidic Acid Methyl Ester
C22:1n9 = Erucic Acid Methyl Ester
C22:0 = Behenic Acid Methyl Ester

Identification of fatty acids methyl esters (FAME)

The taxonomy of filamentous fungi is mainly based on morphology, combined with some physiological and nutritional tests. This taxonomy is often unsatisfactory both because the fungi show considerable morphological variation within established groups and because it has not always provided well defined differences between accepted and postulated species. Several genetic and chemotaxonomic studies have been made in an attempt to remedy this problem, including examination of fatty acids content. Determination of fatty acids has proved one of the most useful tools in bacterial and yeast taxonomy. Published studies of fatty acids in filamentous fungi have suggested that sufficient differences exist to make this approach worthwhile.

The acidophilic fungal strains with high hydrocarbon removal efficiency (RF2, RF3, RF9, RF12, and RF 17) were examined for membrane (free) fatty acids contents. Fatty acids are analyzed as their methyl esters by a GC/FID technique. At first, mycelium was produced in a rich liquid medium, filtered, washed with redistilled water and placed in 50 mL of chloroform:methanol (2:1; v/v) at 60^{o}C for 2 hrs. After filtration, the solvents were evaporated under a stream of nitrogen (Mingrone *et al.*, 1988). One hundred microlitres of mycelium extract were transesterified by boron fluoride (BF_3; 10% in methanol w/v) in 2 mL of methanol and boiled at 60^{o}C for 30 minutes (Zweig & Sherma, 1974; Moss *et al.*, 1980). One milliliter of distilled water was added and the fatty acid methyl esters were extracted three times, each time using 10 mL of *n*-hexane containing 2-3 grams of anhydrous sodium sulphate to remove residual water. The extract was evaporated using a stream of nitrogen. The sample was redissolved in 200 μL of *n*-hexane and 2 μL of the sample was injected into a gas chromatograph with a flame ionization detector (FID). The standards were the mixtures of saturated and unsaturated fatty acids methyl esters purchased from Sigma. The amount of each fatty acid was expressed as a percentage of the total content of fatty acids in the biomass.

The FAME results (means and standard deviations calculated from three repetitions) for five fungal strains with high hydrocarbon removal efficiency are presented in Table 12. Generally, except for myristoleic, *cis*-1-eicosenoic and erucic acids, the differences in the contents of the other fatty acids were statistically significant (ANOVA test). The highest contents were measured for elaidic, oleic, linoleic, palmitic, linolenic, and stearic acids. The *Penicillium* sp. I strains (RF9 and RF12), with the highest hydrocarbon removal efficiency, showed the highest contents of fatty acids. This especially concerns the contents of elaidic, oleic and linoleic

unsaturated acids. The strain RF2 (*Arthrographis* sp.) differed from the other strains in the highest content of palmitic acid while the strain RF3 (*Exophiala* sp.) displayed the highest percentages of linolenic and stearic acids.

Additional characterization of the fungal community from the lagoons at the refinery

Besides acidophilic fungal isolates, some pH-tolerant fungi of particular scientific importance were recorded from soil mixture with sludge at the refinery. Using the hair baiting method (Vanbreuseghem, 1952), over 100 strains were isolated from this mixture. Most of these strains were determined as *Trichophyton ajelloi,* with fast-growing colonies, no microconidia and numerous cigar-shaped, multi-celled, smooth- and thick-walled macroconidia produced (Figure 4). However, six strains displayed characteristics different from those of *T. ajelloi*. These strains grew slowly on test media and produced abundant microconidia and rare macroconidia which were spindle-shaped, thin-walled and verrucose (Figure 5). The strains were preliminarily classified to the genus *Microsporum,* with clear similarity to the *M. gypseum-fulvum* complex. To precisely identify the strains, sexual fruiting bodies (ascomata) were to be obtained. The fungi examined are heterothallic. This means that two (+) and (-) mating strains are required for production of ascomata and crossing experiments using mating type strains of known species are necessary. In order to perform the crossing experiments, mating type strains of *Arthroderma fulva, A. gypsea* and *A. incurvata,* with anamorphs belonging to the *M. gypseum-fulvum* complex, were ordered from the Centraalbureau voor Schimmelcultures (CBS) at Baarn (The Netherlands). These strains were received and the crossing experiment on minimal solid media was performed. Our six *Microsporum* strains did not produce ascomata with any *Arthroderma* testing strains. This indicates that our strains belong to another species. They are suspected to be a rare fungus, *Microsporum ripariae*. To confirm this suspecting by comparing and crossing with our strains, one known strain of *M. ripariae,* isolated from soil in Czechoslovakia in the 70s was ordered from the University of Alberta Mold Herbarium and Culture Collection (Canada). However, the strain has not been received yet. The fungi under examination belong to the group of so-called geophilic dermatophytes, for which keratinolytic activity is characteristic. Apart from this keratinolytic activity, however, the strains also degraded petroleum hydrocarbons, with large biomass production, obviously as a result of long adaptation to the lagoon conditions at the refinery. Also, some results on germination in a drop of petroleum and on growth on media containing petroleum are already available for *Microsporum* strains. The germination hyphae

easily penetrate the drop of petroleum (Figure 6). The fungus produces numerous spiral hyphae while growing on petroleum (Figure 7).

Figure 6. *Trichophyton ajelloi*, the predominating dermatophyte at the refinery's acidic slurry lagoons

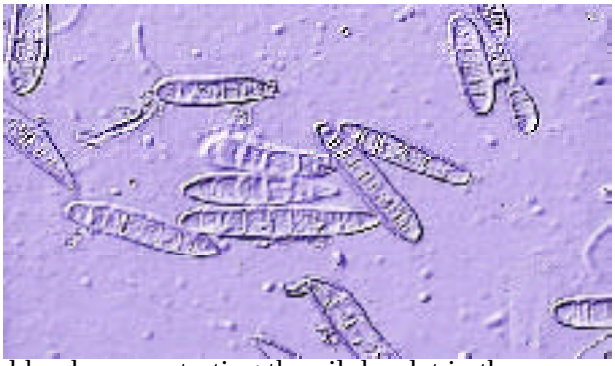
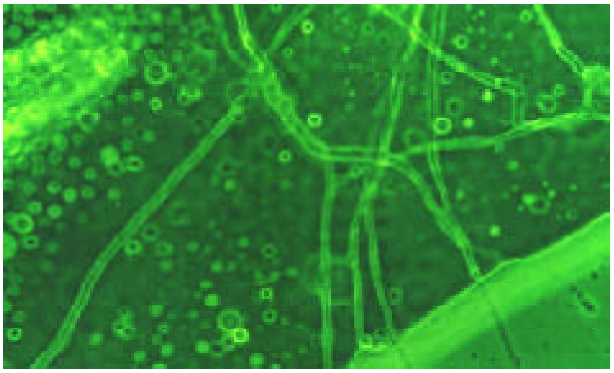


Figure 7. Fungal hyphae penetrating the oil droplet in the presence of proteins



Conclusions

The conclusions are as follows:

1. The acidophilic microbial community from the sludge lagoons at the refinery is exclusively fungal and consists of yeasts and filamentous fungi.
2. Yeasts are more common than filamentous fungi in the acidophilic fungal community. Among the yeasts, *Candida saitoana* along with *Rhodotorula glutinis* are the predominating species while within the filamentous fungi *Pseudozyma* sp. and *Penicillium* spp. are the most common microorganisms.
3. The strains of *Penicillium* and *Exophiala* sp. have the highest petroleum hydrocarbon removal rates and biomass production while the lowest values of these parameters are observed in the *Rhodotorula* strains.

4. The fungal daily growth rates are the highest at pH 2.5 and decrease with increasing pH. However, most of the strains examined are able to grow even at pH 8. The acidophilic fungal community can be, therefore, considered as pH-tolerant rather than strictly acidophilic.
5. The highest daily growth rates are observed at 29^{o}C, with only some strains able to grow at 45^{o}C. The acidophilic fungal community should be, therefore, considered as mesophilic, with good growth at temperatures between 20-37^{o}C.
6. The homogeneity of the constitutive enzyme spectra obtained with the API ZYM bioMèrieux system characterizes the strains of *Candida saitoana*, *Rhodotorula glutinis* and *Pseudozyma* sp. while the spectrum of enzymes for the *Penicillium* strains is rather heterogeneous. This suggests that the *Penicillium* strains belong to two species.
7. The heterogeneity of the *Penicillium* strains is confirmed by the analysis of membrane fatty acids measured as their methyl esters (FAME). The highest quantity of fatty acids is produced by some of the above-mentioned strains, with the highest hydrocarbon removal rate and biomass production. The FAME results may be a useful taxonomic and physiological tool.
8. The acidophilic fungal strains produce a restricted number of extracellular enzymes on solid media. All strains produce catalase and phosphatase while no protease, DNA-ase, CMC-ase, pectinase and polygalacturonase activities are observed. The majority of the strains examined produce lipase, amylase and urease. The lowest number of enzymatic activities (catalase and phosphatase) was noticed in all strains of *Candida saitoana*.
9. Due to the extreme heterogeneity of environmental conditions, the sludge lagoons at the refinery are inhabited not only by the acidophilic and hydrocarbon-degrading fungal strains but also by other physiological groups of fungi, including keratinolytic species.
10. The keratinolytic fungal community at the refinery's lagoons is of special scientific significance. This community consists of the so-called geophilic dermatophytes that apart from keratinolytic activity are also able to degrade petroleum hydrocarbons. Among the dermatophytes, some strains of *Microsporum* appear to be a rare species, *M. ripariae*.

Summary

The present project was to thoroughly characterize the acidophilic and petroleum-degrading microbial community at the refinery's sludge lagoons from molecular, physiological, and taxonomic points of view. This community is exclusively fungal and consists of yeasts and filamentous fungi. Yeasts are more common than filamentous fungi in the community. Among

the yeasts, *Candida saitoana* along with *Rhodotorula glutinis* are the predominating species while within the filamentous fungi *Pseudozyma* sp. and *Penicillium* spp. are the most common fungi. The strains of *Penicillium* spp. and *Exophiala* sp. have the highest petroleum hydrocarbon removal rates and biomass production while the lowest values of these parameters are observed in the *Rhodotorula* strains. The fungal daily growth rates are the highest at pH 2.5 and decrease with increasing pH. However, most of the strains examined are able to grow even at pH 8. The fungal acidophilic community can be, therefore, considered as pH-tolerant rather than strictly acidophilic. The highest daily growth rates are observed at 29^{o}C, with only some strains able to grow at 45^{o} C. The acidophilic fungal community should be, therefore, considered as mesophilic, with good growth at temperatures between 20-37^{o}C. The homogeneity of the constitutive enzyme spectra obtained with the API ZYM bioMèrieux system characterizes the strains of *Candida saitoana*, *Rhodotorula glutinis* and *Pseudozyma* sp. while the spectrum of these enzymes for the *Penicillium* strains is rather heterogeneous. This suggests that the *Penicillium* strains belong to two species. The heterogeneity of the *Penicillium* strains is confirmed by the analysis of membrane fatty acids measured as their methyl esters (FAME). The highest quantity of fatty acids is produced by some of the above-mentioned strains, with the highest hydrocarbon removal rate and biomass production. The FAME results may be a useful taxonomic and physiological tool. The acidophilic fungal strains produce a restricted number of extracellular enzymes on solid media. All strains produce catalase and phosphatase while no protease, DNA-ase, CMC-ase, pectinase and polygalacturonase activities are observed. The majority of the strains examined produce lipase, amylase and urease. The lowest number of enzymatic activities (catalase and phosphatase) was noticed in all strains of *Candida saitoana*. Due to the extreme heterogeneity of environmental conditions, the sludge lagoons at the refinery are inhabited not only by the acidophilic fungal community but also by other physiological groups of fungi, including keratinolytic fungi. The keratinolytic fungal community at the refinery's lagoons is of special scientific significance. This community consists of the so-called geophilic dermatophytes that have keratinolytic properties and are also able to degrade petroleum hydrocarbons. Among the dermatophytes, some strains of *Microsporum* appear to be a rare species, *M. ripariae*.

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Project Deliverable

Bioreactor Project

FY00 Final Report – Task 2

Production-Scale Implementation of Petroleum Contaminated Soils Bioreactor

Submitted to

U.S. Department of Energy

National Energy Technology Laboratory

Morgantown, West Virginia

Submitted by:

Institute for Ecology of Industrial Areas

Katowice, Poland

Institute for International Cooperative Environmental Research

Florida State University

Tallahassee, Florida, USA

November 2000

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Executive Summary

This project was a cooperative research initiative among the Institute for Ecology of Industrial Areas (IETU), Katowice, Poland, the Westinghouse Savannah River Technology Center (SRTC) and Florida State University. The project was sponsored by and conducted for the United States Department of Energy (DOE). The project is managed under the DOE EM-50-Joint Coordinating Committee for Environmental Systems (JCCES) agreement.

Petroleum contaminated soils, on one scale or another, are common to all DOE sites as well as those of other government agencies and commercial locations worldwide. While large areas of contaminated soils justify dedicated remedial operations, smaller areas could be addressed with *ex situ*, on-site batch remediation. This approach also has been proposed for removing organic contaminants from low-level mixed wastes, thus allowing the waste to be disposed of as a low-level waste, a much simpler situation than that of mixed waste. This approach would satisfy not only the ability to deal with relatively small volumes of waste material but would support relatively stringent clean-up standards. Highly controlled, small-scale operations would be well suited to such an activity and could be used widely throughout the DOE complex and in other locations worldwide.

A preceding project, "Bioremediation of petroleum contaminated soil at the Czechowice Oil Refinery" was successfully completed in 1999 and provided the basis for this project. Implementation of this technology for the remediation of petroleum contaminated soils at the refinery reduced human health and environmental risk while establishing a green zone within the refinery. Bioremediation was demonstrated to be a useful technology at the refinery through a comprehensive performance monitoring effort. In the course of the refinery project, 81% of the initial TPH content in the contaminated soil was removed.
Recognition of the widespread nature of the petroleum contaminated soils problem along with the experience gained during the realization of the Czechowice Oil Refinery Project were driving factors behind further efforts to optimize and enhance the bioremediation process.

The goal of this project was the design, construction, start-up and operation of a small, mobile bioreactor. This continuous airflow, packed bed reactor was used to remediate the small volumes of contaminated soils or investigatively derived wastes (IDW).

The bioreactor consists of:

(1) modified 6 m^3 capacity standard container for waste collection and transport ("roll off"). The container is designed and modified to provide an air-tight environment. The covers, through which the bioreactor can be loaded/unloaded are sealed with rubber gaskets. The bioreactor is equipped with a false floor to provide leachate collection and uniform aeration of the target soil;

(2) supplementary equipment to control air flow, nutrient availability and moisture level. This includes blowers to force airflow through the soil layer as well as pumps and sprinklers to recycle the leachate from the bed back to the process. The leachate recirculation system provides a mechanism for the uniform distribution of fertilizer or other system amendments; and

(3) monitoring system which allows continuous control of the process through a system of sensors that monitor the composition of the air flowing into and out of the bioreactor, soil air composition, soil moisture and temperature.

The proposed design is expected to provide maximum flexibility for application to many sites for rapid *ex situ* bioremediation of a variety of organic contaminated soil.

Once the bioreactor was built, soil cleanup tests were carried out. Approximately 3.2 tons of petroleum contaminated soil from Czechowice Refinery was amended with wood chips and fertilizers and placed in the bioreactor for bioremediation. Bioremediation tests lasted 97 days. Bioreactor operations went according to plan. During operational tests, both TPH and PAH were reduced to ~50% of initial concentration. Results are comparable to results obtained during previous column tests conducted for the refinery Biopile Project. Results obtained indicate that bioreactor construction and data monitoring system combined should provide a useful tool to biormediate limited amounts of petroleum contaminated soil.

Introduction

Petroleum contaminated soils, on one scale or another, are common to all DOE sites as well as to those of other government agencies and commercial locations worldwide. While large areas of contaminated soils justify dedicated remedial operations, smaller areas could be addressed with ex-situ, on-site, batch remediation. This approach also has been proposed for removing organic contaminants from low-level mixed wastes at the Savannah River Site, thus allowing the waste to be disposed of as a low-level waste, a much simpler situation than that of mixed waste. Such an approach would satisfy not only the ability to deal with relatively small volumes of waste material, but would support relatively stringent clean-up standards. Highly controlled, small-scale operations would be well suited to such an activity and could be used widely throughout the DOE complex and other locations worldwide.

Bioremediation is a promising technology for removing organic contaminants from soil. The process mineralizes or transforms hydrocarbons (both xenobiotic and naturally occurring) introduced in the environment to less toxic or innocuous forms (Atlas, 1984). Many microorganisms including bacteria, fungi, yeast and algae have enzymatic capacity to completely mineralize petroleum hydrocarbons and utilize carbon components to generate new biomass. Indigenous microorganisms in soil and groundwater can degrade large quantities of petroleum hydrocarbons if they are provided sufficient amounts of water, oxygen, and other limiting nutrients, usually nitrogen and phosphorus. On-going research conducted by the Institute for Ecology of Industrial Areas (IETU) at the Czechowice Oil Refinery (CzOR), has demonstrated significant advances in technology. These advances have resulted in reduction of cost, time and complexity while improving the efficiency of bioremediation for soils contaminated with petroleum hydrocarbons.

Further refinements are needed in order to optimize applicability of this technology. Areas requiring continued research and development include:

- better control of bioremediation physical parameters (e.g. temperature, moisture, water, air and nutrient flows),
- methods for designing/utilizing a bioreactor as batch processing unit capable of being reused for *ex situ*, but on-site treatment of contaminated soils, and

- the potential role of surfactants in degradation of recalcitrant compounds under batch processing conditions.

Soil bioventing conducted in a batch reactor (bioreactor) appears to be a proper means to achieve above-mentioned goals. Bioreactors are most frequently mobile treatment units, easily moved on and offsite when necessary. ioreactor clean-up time is relatively short, with times ranging between 2 and 24 months. During treatment, personnel are not required to be onsite full time thus limiting exposure and cost. Finally, remediation with bioreactors is rather inexpensive compared to other types of possible remediation processes, because the system is reusable.

Project objectives are to develop design criteria, construct a bioreactor and identify limiting operational parameters. Determination of time factors that would optimize bioreactor operation is another project objective.

Treatment design incorporates advances gained in the CzOR project (e.g., leachate recirculation/moisture level, temperature control needs, nutrient demands and supply techniques), and implementation and evaluation of innovative, field deployable *in situ*, autologging, data sensors as well as evaluating design parameters needed to optimize the bioremediation of contaminated soils.

Bioreactor description

Background/Purpose

Proposed treatment consists of a small, mobile bioreactor, designed as continuous airflow, packed bed reactor. The SRTC proposed design, which is used as a reference, specifies two different reactor sizes, one for smaller quantities of material (nominally 10 ft^3) and a "skid pan" design for larger quantities of material (nominally 6 yd^3). Both of these reactors utilize the bioventing process.

Discussion and description of facilities and bioreactor design include the following issues:

_ description of reactor system and process variables,

_ technique of measurement,

- example of design calculations (TPH degradation rate, range airflow rates, gas emission, pressure drop, treatment time or reaction time), and
- description of process (flowsheet, schematics, leachate containment system).

On review of the SRTC reference design, conclusions were drawn:

- IETU has appropriate means to design and operate a bioreactor. Construction needs to be subcontracted to a specialized firm or workshop;
- Monitoring/control equipment should be available locally at prices roughly comparable to US prices;
- As the bioreactor is to be operated at IETU facility, smaller reactor (~300 L) seemed to be a better alternative logistically**.** However during additional discussions with SRTC it was decided that this is too small. A larger, highly instrumented, commercial/production unit (2 m^3 minimum volume) would be constructed; and
- Completions of project goals have been defined as: construction of reactor and collection of operation data of the unit (including evaluation of sensor performance and applicability).

Bioreactor design and construction

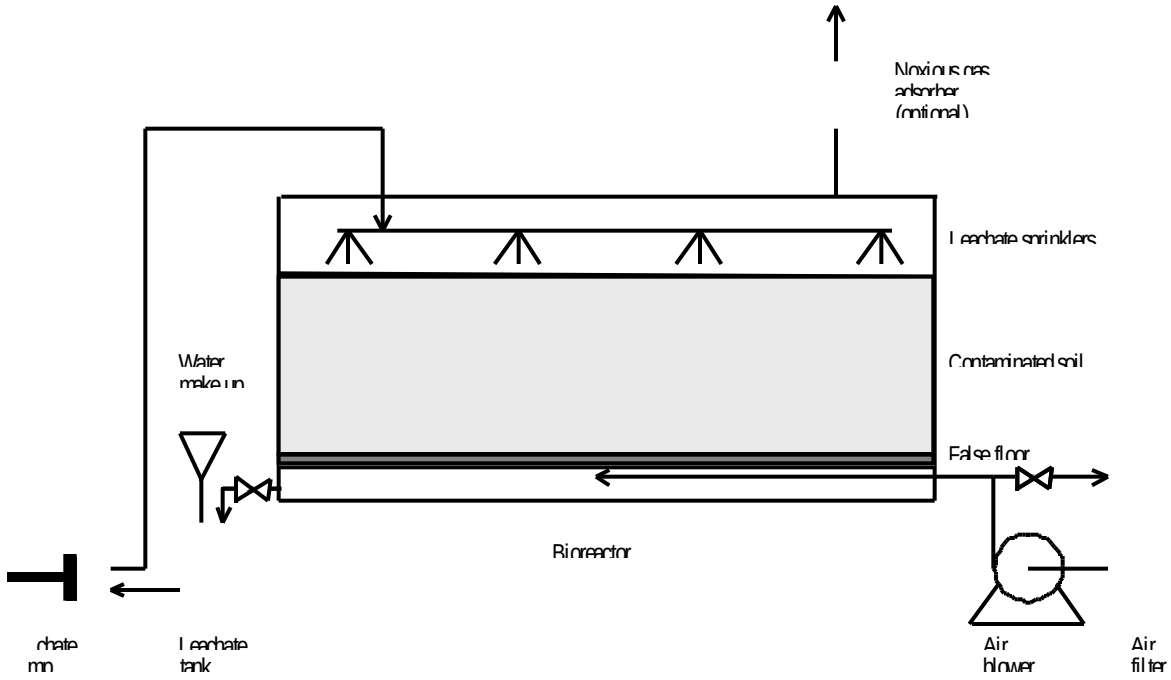


Figure 1. Bioreactor aeration and leachate circulation system

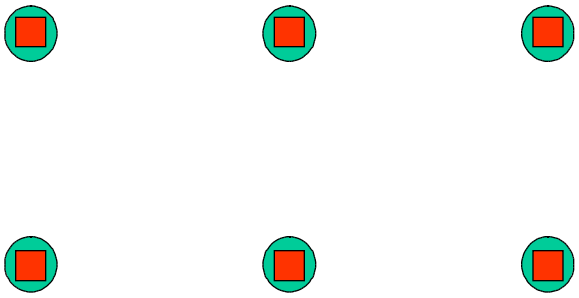


Figure 2. Measuring and sampling points diagram.

Reactor system description

A bioventing process was realized in the bioreactor system consisting of:

- bioreactor,
- aeration system,
- leachate circulation system, and
- monitoring system.

The bioreactor (Figure 1) is designed as a continuous airflow, packed bed reactor. An adapted regular waste container (a “skid pan”) with a nominal volume of 6 m^3 (3.50 m long, 1.73 m wide and 1.00 m deep) was used as a vessel where contaminated soil was bioremediated. The adaptation included:

- continuous welding of walls and vessel cover modification to ensure air-tightness of the reactor vessel (regular skid pan is not tight enough);
- mounting of false floor; and
- providing necessary connector pipes, valves, sprinklers etc. for aeration and leachate circulation systems.

The aeration systemconsists of an air blower equipped with an inlet air filter, a by-pass pipe equipped with a control valve, an inlet air nozzle, and (optionally) a noxious gases adsorber at the bioreactor gas outlet.

The leachate circulation system includes a drain equipped with a valve, a small tank vessel, a pump and a system of sprinklers.

The whole system has been equipped with necessary devices placed in several sampling ports (Figure 2) for monitoring main parameters of bioremediation.

Bill of materials

Bioreactor

Regular waste container ("skid pan"), 6 m^3 volume	1 piece
Air compressor Orion OL 200 with a 50 L surge tank, producer: BALMA (Italy)	1 piece
Metering pump Normados N-P31, capacity 20 L/hr, producer: Bran Lübbe (Germany)	1 piece
Zinc coated platform grid	6 m^2
Angle bars: 40 x 40 x 4 mm	48 kg
45 x 45 x 4 mm	70 kg
60 x 60 x 6 mm	9 kg
Channel bar 65 mm	50 kg
Steel sheets: 1.5 mm	72 kg
2.5 mm	40 kg
5.0 mm	160 kg
Rubber sheet 10 mm	20 kg
Aluminum rivets 5 x 12 mm	0.35 kg
Bolts M-8	3 kg
Nuts M-8	2 kg
Rubber – metal glue	1 L
Silicon leaching stopper	1.5 L
Polyester hard putty	0.5 kg
Quick-release joint 3/8"	1 piece
Ball valves:	
1/2"	2 pieces
3/4"	1 piece
Priming paint	2 L
Plastic hose 3/8" equipped with quick-release joints	5 m
Steel pipe 1/2"	8 m
Fittings, couplings, etc. for leachate sprinkling system	1 set

Geotextile to cover the bioreactor false floor was provided by SRTC

Measuring and data acquisition equipment

O_2 soil gas/air sensors DRC-XT253	
producer: Microbac (US)	8 pieces
Hydrocarbons soil gas/air sensor DRC-ADS201	
producer: Microbac (US)	8 pieces
CO_2 soil gas/air sensor GMM220	
producer: Vaisala (US)	3 pieces
Soil temp sensor DRC-TKO24	
producer: Microbac (US)	6 pieces
Soil gas humidity sensor	6 pieces
Air temperature and humidity sensor EE-15	
producer: Introl (PL)	2 pieces
Gas flow meter BK-6	
producer: Intergaz (PL)	2 pieces
Signal conditioning system, IETU design	1 set
Data acquisition, PC card PCL-812PG/720	
producer: Introl (PL)	1 piece
Data acquisition, ADAM Modules	
producer: Advantech (US)	1 set
Cables, couplings etc.	1 set

Design calculations

Stoichiometry

Assume that H/C ratio in a hydrocarbon contaminant is m/n. Thus, the stoichiometric relationship determining the oxygen demand for contaminant degradation takes the following form:

$$C_nH_m + \left(n + \frac{m}{4}\right)O_2 = nCO_2 + \frac{m}{2}H_2O$$

and mass ratio of the contaminant to oxygen (S) is:

$$S = \frac{12n + m}{\left(n + \frac{m}{4}\right)32} = \frac{1}{8}\left(\frac{12 + \frac{m}{n}}{4 + \frac{m}{n}}\right)$$

According to this equation, S depends on H/C atomic ratio only. It changes over the entire range of hydrocarbons: for methane (a hydrocarbon richest in hydrogen) S = 0.25,lowest S-value, whereas for pure carbon S = 0.375 highest possible S-value (see Figure 3). Its reciprocal indicates how much oxygen is needed to degrade 1 g of contaminant.

Assume that in model particles of contaminant roughly half of C atoms are in aromatic form (with H/C ratio as 1:1) and half in saturated aliphatic form (H/C ratio being 2:1) resulting in overall H/C ratio of 1.5:1 where S is equal to 0.31. This value is used in the Test Plan.

Required air flow rate

Assumptions made to calculate required air flow rate include:

- peak rate of hydrocarbon contaminant biodegradation: 1,000 mg/kg of soil/day,
- concentration of oxygen outlet: 5 % vol., and
- estimation of soil bulk density: 1.6 kg/dm^3.

From stoichiometry (see above) it follows that during biodegradation of 1000 mg/kg/day of a hydrocarbon, 1000/0.31 = 3 226 mg $O_{2/}$kg/day = 3.23 g $O_{2/}$kg/day of oxygen is consumed. The bioreactor contained ~ 5 metric tons of soil. Thus, the total oxygen consumption rate is: 3.23 g/kg/day x 5 000 kg = 16 150 g $O_{2/}$day = 16.15 kg $O_{2/}$day. At normal pressure and temperature conditions it gives: 16.15 x 22.4/32 =11.3 m^3 $O_{2/}$day. Taking into consideration required outlet oxygen concentration, air flow rate can be calculated: 11.3/0.15 = 75.33 m^3/day or 3.13 m^3/hr of air. In pressure drop calculation a value of 6 $m^{3/}$hr was taken for air flow rate.

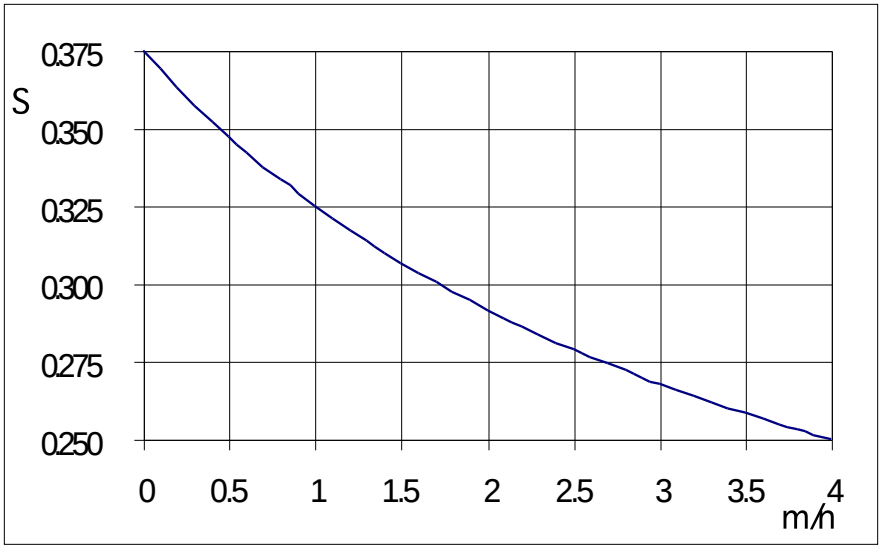


Figure 3. Dependency of S on m/n ratio.

Pressure drop

A modified Darcy-Weisbach equation for compressible fluids was used to calculate pressure drop across the bioreactor soil bed:

$$Q\,p_a = \left[\frac{p_a{}^2 - p_b{}^2}{2} + \left(\frac{p_a + p_b}{2}\right)^2 \frac{Mg}{RT}\right]\frac{KA}{\eta L}$$

Where:

Q - fluid volumetric rate

p_a, p_b – fluid pressure at inlet and outlet to the bed, respectively

M – molar mass of the fluid

g – gravity acceleration

R – universal gas constant

T – temperature

K – soil permeability

A – flow cross-section area

η - dynamic viscosity coefficient of the fluid

L – height of the bed

Any consistent system of units can be used in calculations.

A plot of the Darcy-Weisbach equation illustrates pressure drop dependency on air

surface loads for different K values (Figure 4). K was assumed as equal to 100 mDa (10^{-13} m^2).

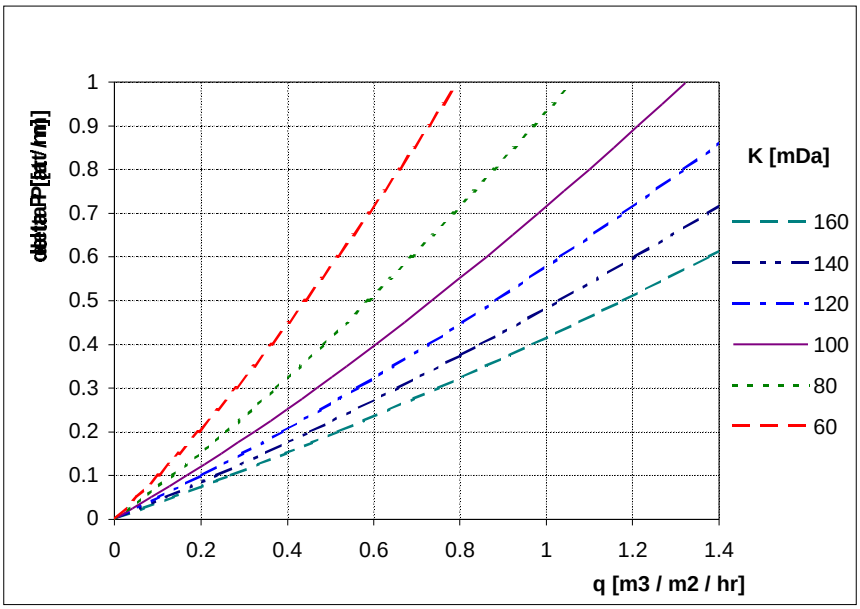


Figure 4. Pressure drops vs. air surface load.

For Q = 6 $m^{3/}$hr and A = 6 m^2 one obtains q = 1 m^3 /m^2 /hr and delta p = ~0.72 at/m. As the soil bed height was 0.5 m, the pressure drop across the bioreactor soil bed is expected to be ~0.36 at.

Treatment time or reaction time

Required reaction time or batch "holding time" (treatment time) is based on starting contaminant concentration, rate of hydrocarbon degradation and "target levels".

In engineered systems such as bioreactors, diffusion of oxygen from the atmosphere into soil can be reduced compared with diffusion in free air as a result of the tortuous and some time blocked diffusion paths through soil pores. But, generally, it remains much faster than transport through water phase. Thus, if soil water content is kept sufficiently low, oxygen availability is highly unlikely to control biodegradation processes.

Bioreactor inventory is soil-water system dominated by solid surfaces to which microbes are attached. Bacteria, with a typical size of about 1 μm, are excluded from entering

smaller micropores of soil and porous media. External enzymes produced by microorganisms are, as a rule, much larger than contaminant molecules and microbially produced surfactants are roughly the same size as contaminants. In consequence, diffusion coefficients of surfactants are lower than those of contaminants. Thus, pollutants in micropores of soil aggregates or solids may be unavailable to bacteria and must diffuse through pore water to external grain surfaces to be degraded. Intrapore diffusion may be retarded by sorption of pollutants to solid surfaces of micropores. In many cases, pollutant mass sorbed on solid surfaces is largely unavailable to microbes. Thus, a working hypothesis for aerobic solid-water systems considers primarily mobile, aqueous-phase substrates to be bioavailable for microbial degradation. When non-aqueous-phase liquids (NAPL) are present in the system, only the fraction of pollutant mass dissolved in aqueous phase may be assumed to be bioavailable. Another factor that affects bioavailability of organic pollutants in porous media is aging, i.e., length of time soil has been exposed to contamination. As contaminated soil ages, pollutants may diffuse into smaller, more tortuous micropores and chemically bind with soil, becoming increasingly recalcitrant to biodegradation over time. Aging may result in weathered films that inhibit dissolution of complex NAPLs such as crude petroleum and acid refining wastes.

In many cases (e.g. Czechowice Oil Refinery Bioremediation Project) contaminant mass transfer is the rate limiting step. In such situations, attempts to increase biodegradation rates by creating optimal conditions for microbial activity cannot be effective. However, application of surfactants can increase rate of contaminant removal, but it is not a hard and fast rule. Generally, soils with high concentrations of clay and/or organic matter and containing aged contaminants seem to be unsuitable for bioreactor operation. A three month test was planned to determine actual effects of these factors.

Process Description

Process Variables

The suggested range of bioremediation operating parameters (Kastner *et al*., 1998) is shown in Table 1.

Table 1. Suggested ranges for design parameters in the bioremediation of petroleum

contaminated soil. Suggested ranges for design parameters in the bioremediation of petroleum contaminated soil.

Parameter	Range	Method of Addition	Comments/Monitoring
Oxygen concentration	5-21 %	Air injection	Test effect of aeration via measurement of TPH elimination in the soil, Periodic O_2 uptake measurement, and mass balance on inlet and outlet O_2 Sensor
Soil moisture	30-80% of Field capacity or roughly 8-20% by weight	Sprinkler or irrigation systems	Water mass balance Periodically pull soil samples for oven drying to determine moisture content or use soil Moisture probes
Nutrients: Carbon/Nitrogen/Phosphorus or C/N/P ratio	C/N/P = 100/10/2	Irrigation: water soluble nitrogen and phosphate fertilizers	Periodically measure nitrogen and phosphorous concentrations in soil Soil sampling and analysis
Soil pH	4 to 7	If needed, nutrient addition can act to buffer the soil	Periodically measure pH
Hydrocarbon Level			Periodically measure soil hydrocarbon level. Hydrocarbon sensor verified by periodic soil sample analysis
Soil temperature	20^0 to 45^0C	Ambient Air	Rates would be slowing down during the winter but microbial activity would not cease because of low temperatures Bioremediation has been shown to occur in soil temps. as low as 7^0C and up to 55^0C Thermocouple

Process Monitoring System

A process monitoring system was designed to control bioreactor's working parameters and to evaluate the rate of bioremediaton. To control the mass balance during the bioreactor operation, the following parameters were measured:

- inlet and outlet gas parameters (e.g. gas composition, temperature, pressure, flow rate, etc),
- soil temperature and moisture content, and
- soil gas composition.

Sensors used in bioreactor monitoring system are presented in the following table (Table 2). The sensors were bought from suppliers in the United States (US) and in Poland (PL).

Table 2. Bioreactor monitoring sensors.

Measured parameter	Sensors type	Supplier	Pieces	Description
Oxygen contents	DRC-XT253	Microbac US	8	O_2 contents in soil and outlet gases.
Hydrocarbons contents	DRC-ADS201	Microbac US	8	CH_4 contents in soil and outlet gases.
Carbon dioxide contents	GMM220	Vaisala US	3	CO_2 contents in soil and outlet gases.
Soil temperature	DRC-TKO24	Microbac US	6	Thermocouple type K.
Gas humidity and temp.	EE-15	Introl PL	2	Measure of inlet and outlet gases.
Gas flow meter	BK-6	Intergaz PL	2	Measure of inlet and outlet gases.
Gas pressure	Eco-tronic	Introl PL	1	Measure of inlet air pressure.
Soil moisture	Moisture block	-	6	Gypsum blocks.

Output signals (which differ from one sensor type to another – see Table) from the sensors were logged by a data acquisition system using remote data acquisition modules (delivered by Advantech Inc. US, see Table) connected to a PC computer in which the signals were calculated and stored.

Table 3. Bioreactor data acquisition modules (delivered by Advantech Inc. US).

Module type	Connected sensor
ADAM-4018	DRC-XT253 – 8 channels
ADAM-4017	DRC-ADS201 – 8 channels
ADAM-4017	GMM220 – 3 channels, EE-15 – 2 (temp) + 2 (hum) channels, Eco-tronic – 1 channel
ADAM-4018	DRC-TKO24 – 6 channels
ADAM-4018	Moisture blocks – 6 channels
ADAM-4080	BK-6 – 2 counter channels

The modules use RS-485 (twisted pair) interface to communicate each other. They are connected to PC computer by additional module – ADAM-4522, which includes RS-485 to RS-232 converter. The data acquisition system includes an ADAM-3854 (power relay module) and RP-1072-24 (DC power supplier module).

Table 4. Sensors output signals.

Sensors type	Output signal
DRC-XT253	0 – 60 mV
DRC-ADS201	0 – 6.5 kOhm
GMM220	0 – 5 V
DRC-TKO24	thermocouple type K
EE-15	0 – 10 V
BK-6	digital on/off pulse generator
Eco-tronic	4 – 20 mA
Moisture block	impedance (alternating current)

Since ADAM modules are able to measure voltage and digital signals, only outputs from hydrocarbons sensors (DRC-ADS201) and pressure sensor (Eco-tronic) had to be converted. Conversions are described in detail in Section 0.

Sensors localization

Sensors measuring gas compositions and soil parameters were placed in the bioreactor vessel. Inlet air and outlet gas temperature and humidity are measured outside the vessel in the vicinity of corresponding gas flow meters. Localization of all sensors in the bioreactor is shown in Figure 3 to Figure 6. Six sampling points (SP 0 to 5) have been placed in the soil bed (see Figure 7 and Figure 8). At these points O_2, and hydrocarbons concentrations, temperature and soil humidity were measured. Additionally,

concentration of CO_2 was measured in SP 2 and SP 3 (point placed in unamended soil in the plexiglas column). One sampling point was placed in the vessel above soil bed SP 7. At this point concentrations of O_2, hydrocarbons and CO_2 are measured. Inlet and outlet gas temperature and humidity and inlet pressure were measured outside the vessel in the vicinity of gas flow meters because these parameters were used to recalculate gas flow into normal conditions and should be measured as close as possible to corresponding gas flow measurement points.

Soil gas sensors were shielded with use of a geomembrane to prevent their damage by soil particles. Since working CO_2 sensors produce some heat that may influence operations of other sensors, they were located away from other sensors.

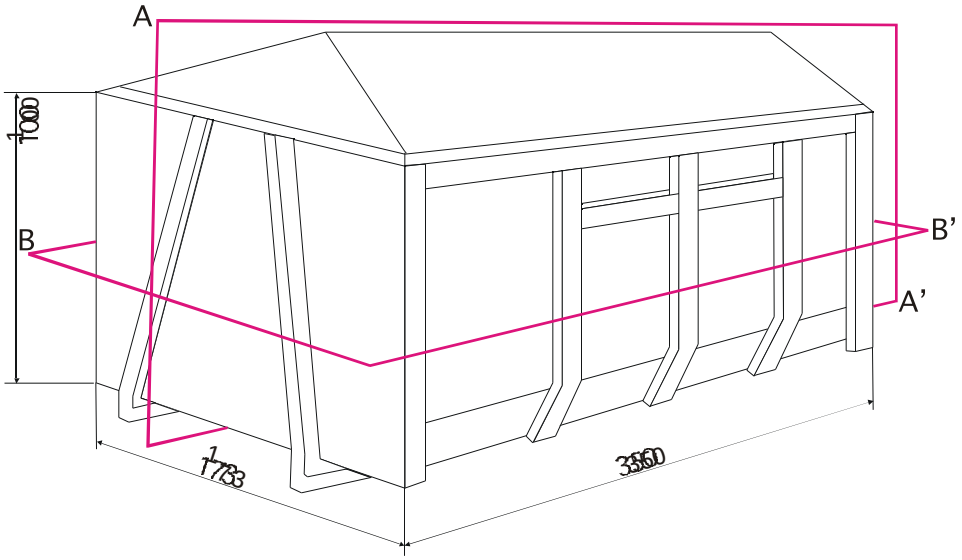


Figure 5. Bioreactor vessel. Thick lines show cross sections presented in next figures. Dimensions are in centimeters.

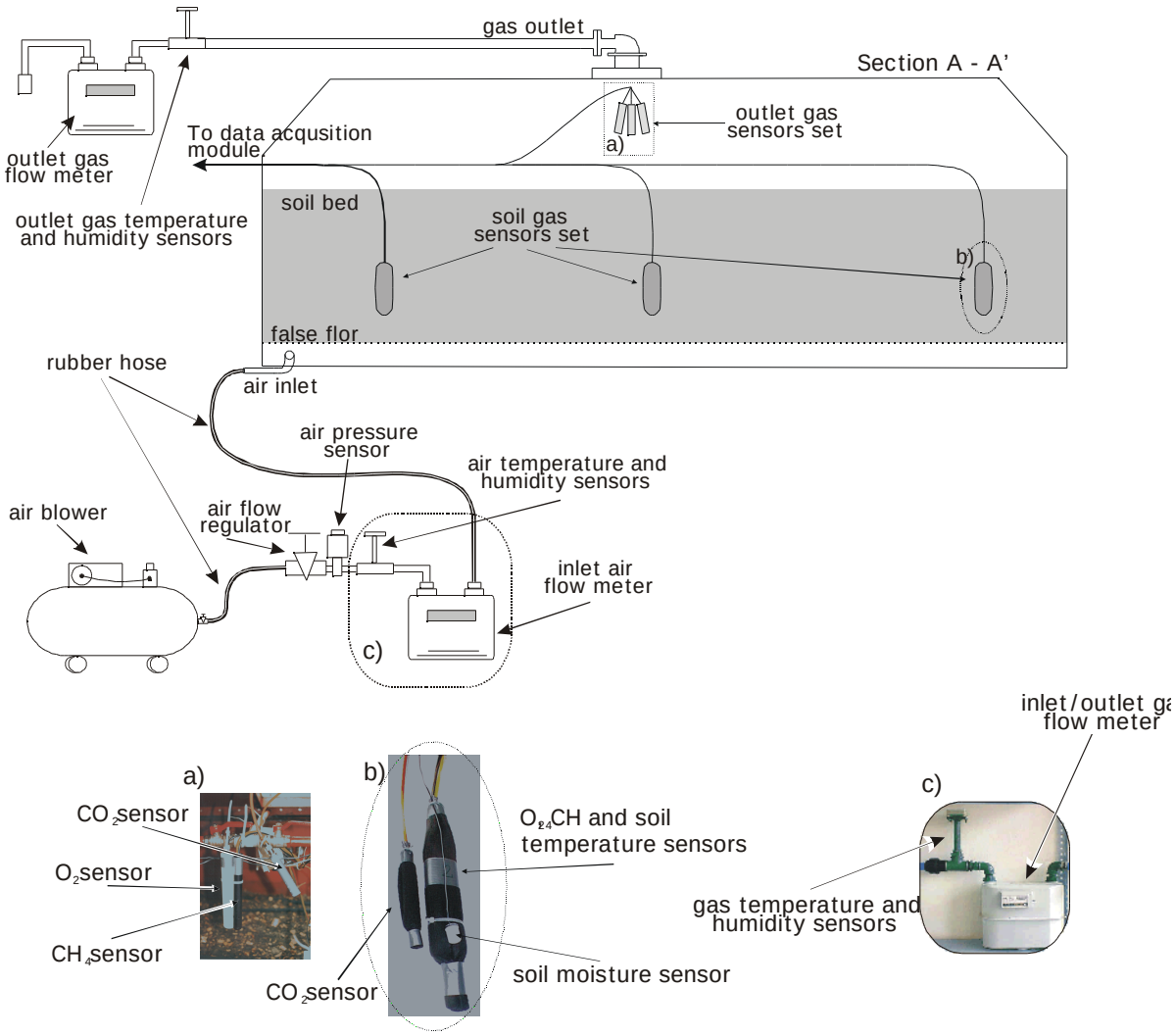


Figure 6. Localization of sensors sets in bioreactor system, vertical section of bioreactor vessel. Inserts show pictures of used sensors sets: a) set placed above soil bed in the bioreactor chamber, b) set shielded by geomembrane and placed directly in soil bed c) gas flow meter with temperature/humidity measurements.

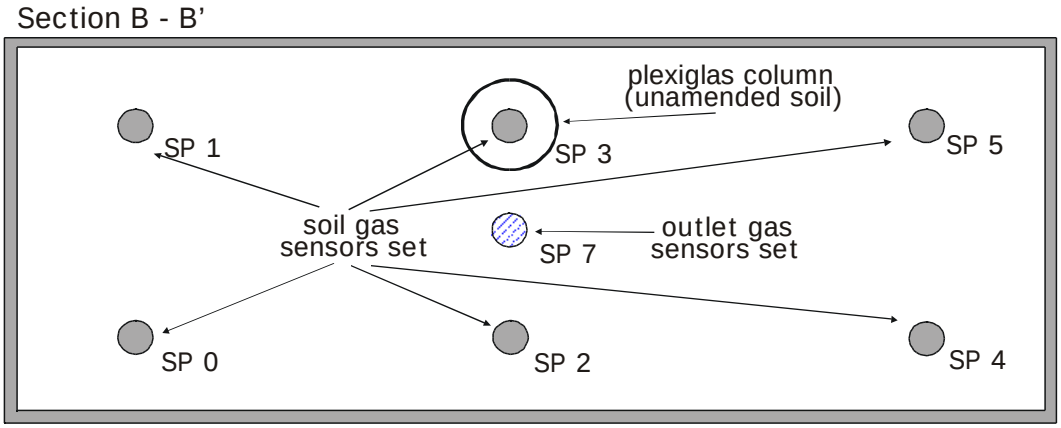


Figure 7. Localization of measuring points in the bioreactor, horizontal section of bioreactor vessel. Sampling points (SP) from 0 to 5 are placed in soil bed. SP 7 outlet gas sampling port is located close to bioreactor outlet above the soil bed.

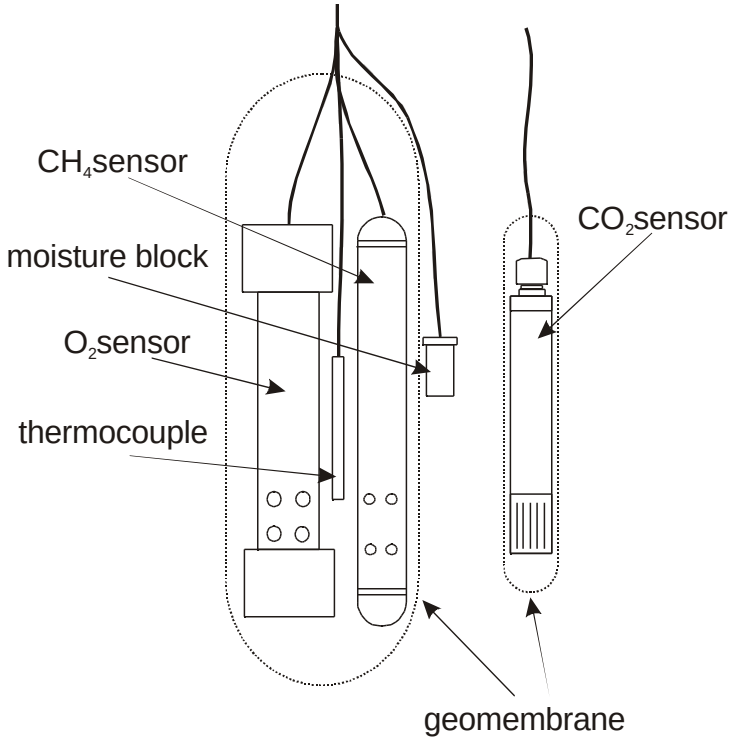


Figure 8. Diagram of sensors set placed in soil sampling point (SP 0 – 5). Oxygen and methane sensors and thermocouple are placed together and shielded by geomembrane. Soil humidity sensor is placed outside geomembrane. Carbon dioxide sensor is separate and also shielded by geomembrane.

Data acquisition system

Data acquisition system was build with use of Advantech Inc. ADAM 4000 series modules and a PC computer running MS Windows NT 4.0 workstation. Output signals from sensors are wired to ADAM-4017 or ADAM-4018 modules (see Figure 9). These modules include 8 channels 16 bits analog to digital converters. Digital pulses from gas flow meters are wired to ADAM-4080 module, which include two channels 16 bits counters. This module is also used to control, via power relay module, power supply of the sensors and constant current source module. It automatically switches sensors on a few minutes before measurement (due to warm up time) instead of powering them all the time. This solution decreases power consumption and increases lifetime of the sensors. All modules are connected together via RS-485 interface and to computer via ADAM-4522 converter an RS-232 interface. Data acquisition process is fully controlled by IETU created software (called BioReDaq) working in Advantech VisiDaq environment (see Figure 10 to Figure 12). Using this computer program it is possible to observe direct output sensors signals, measured values expressed in natural units (e.g. % of volumes, m^{3}/h, etc.) and input parameters (individually for each sensor) used for conversion from output signal to natural units. Using BioReDaq allows also to schedule whole data logging process e.g. sampling intervals, sensors warm up time, and number of samples used to calculate the average logged value. BioReDaq saves all data in ASCI text files, which are then sent to MS Access database system for future calculations and presentations.

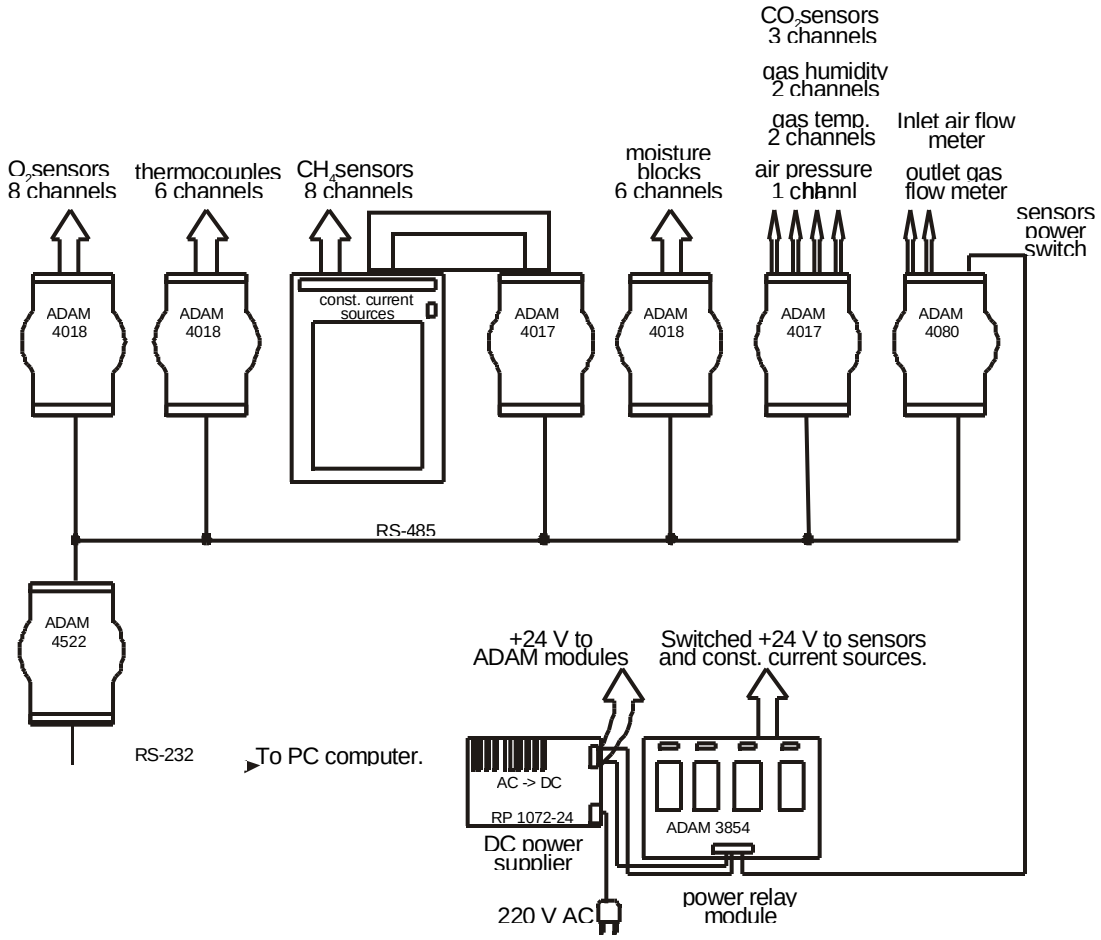


Figure 9. Block diagram of data acquisition system.

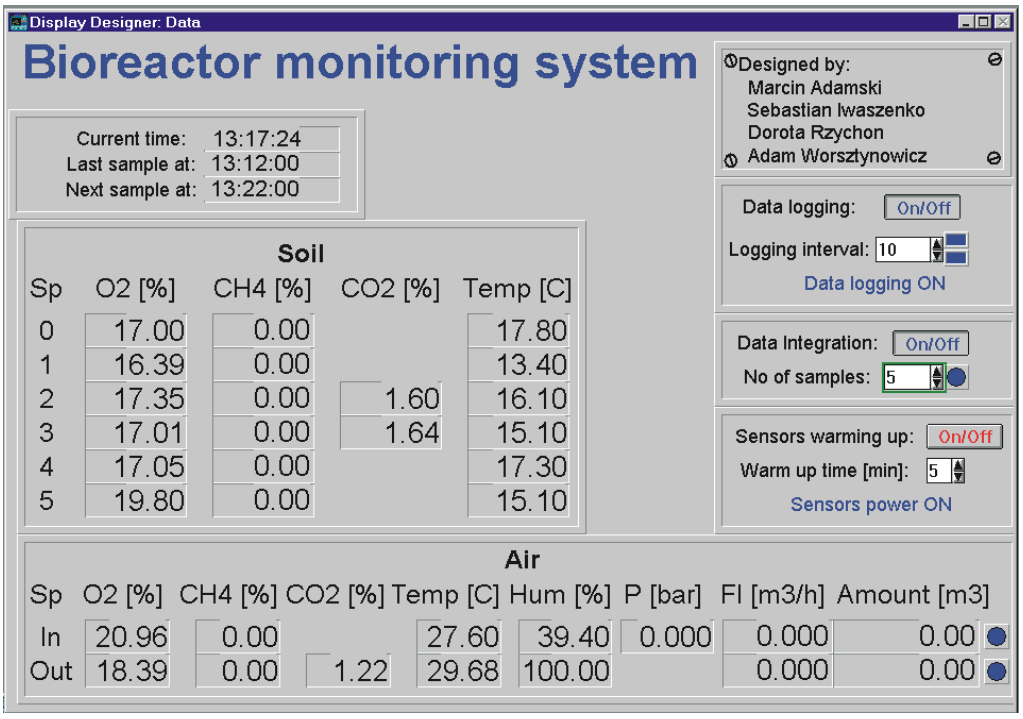


Figure 10. Screen shot of main form of Bioreactor Monitoring computer program BioReDaq.

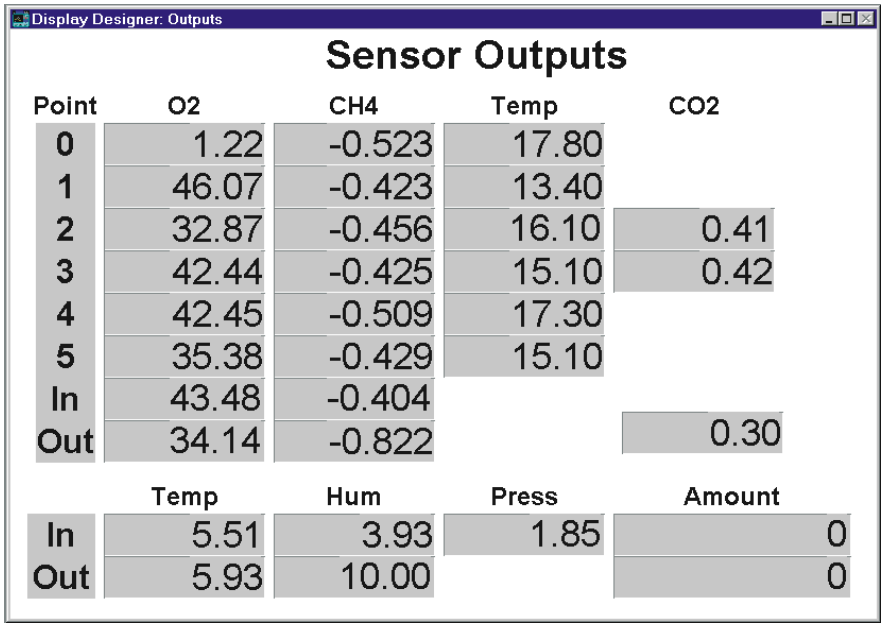


Figure 11. Screen shot of sensors output form of BioReDaq.

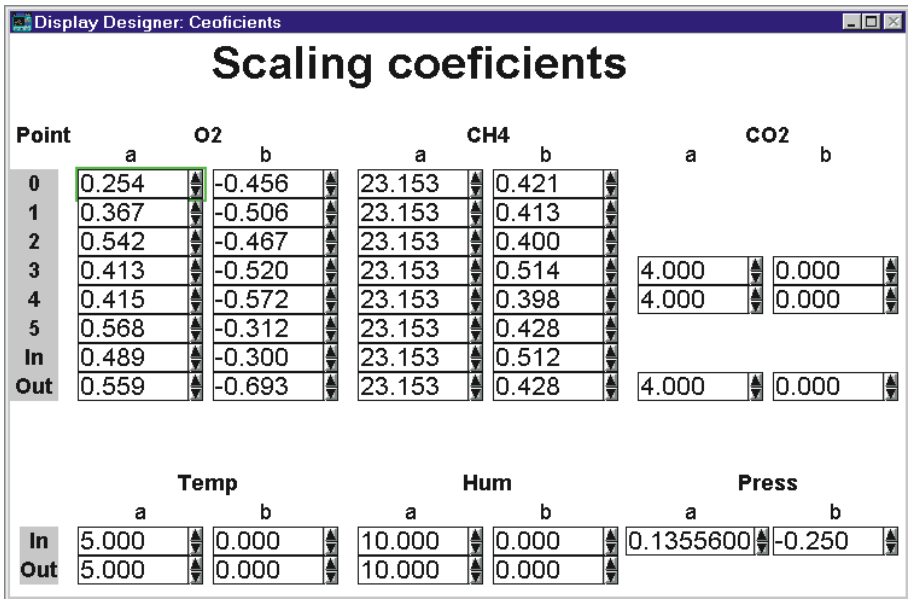


Figure 12. Screen shot BioReDaq form used to input sensors scaling coeficients.

As mentioned, Advantech analog input ADAM modules are able to read voltage signals only. Since methane sensors have resistance outputs, it was necessary to convert them to voltage signals. Conversion was accomplished by using a constant current source module developed at IETU (see Figure 9). Sources are highly temperature stabilized, they have independent DC constant power regulator and are insensitive to voltage changes in the main power supply.

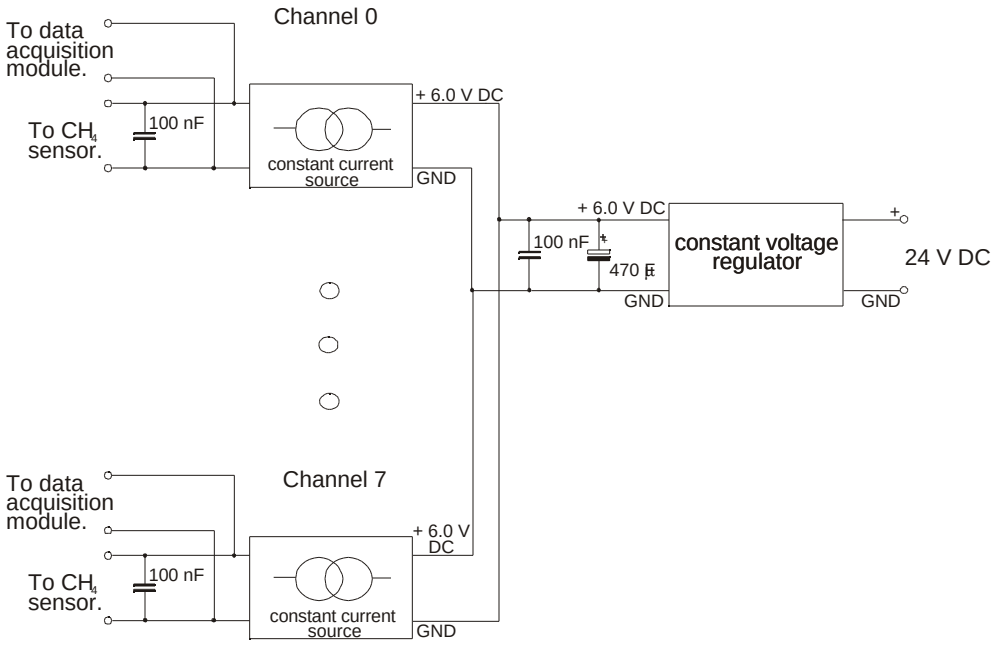


Figure 13. Diagram of constant current sources module.

Petroleum contaminated soil cleanup test description

Soil preparation

A sample (approximately 4 tons) of petroleum contaminated soil from Czechowice Refinery was transported to IETU. Soil was sieved and mixed with about 300 L of oak and pine chips, as well as with 100 kg of Saletrzak (main component: ammonium nitrate) and 50 kg of Poldap (mostly monoammonium phosphate and diammonium phosphate) fertilizers. Prior to adding amendments, approximately 60 kg of sieved soil was separated to serve as a reference sample. Sample points were inserted in both open ends of the plexiglass cylinder in the bioreactor soil bed (see Figure 14). Thus, bioremediation conditions for both amended and unamended soil samples should be similar and comparisons should be possible.

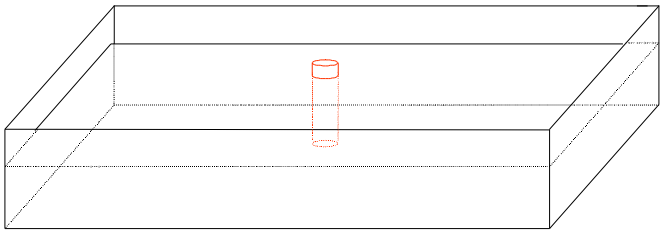


Figure 14. Localization of unamended soil sample in the bioreactor (not to scale).

5 samples of amended soil and 1 sample of unamended soil were taken for chemical and microbiological examination. Results are shown in Table and Table .

Table 5. Chemical analysis of soil at the beginning of bioremediation.

Analysis	Unit	Sampling Point					
		SP 0	**SP 1**	**SP 2**	**SP 3** *	**SP 4**	**SP 5**
pH in KCl	-	6.96	6.94	6.98	6.71	6.88	6.88
pH in H_2O	-	7.07	7.14	7.1	6.99	7.01	7.1
Conductivity	[mS/cm]	7.901	6.263	6.735	0.574	7.913	8.509
$N\text{-}NH_4$	[mg/kg dry soil]	42.23	53.28	45.11	5.28	35.37	43.53
$N\text{-}NO_3$	[mg/kg dry soil]	2057	2329	1151	13.11	980	1414
$N\text{-}NO_2$	[mg/kg dry soil]	4.19	6.05	7.46	8.12	8.41	6.2
TKN	[%]	1.38	1.18	1.11	0.25	1.12	1.23
Total P	[%]	0.41	0.41	0.41	0.07	0.73	0.41
PO_4	[mg/kg dry soil]	3297	1776	1930	168.6	2231	2389
TPH	[g/kg dry soil]	26	27.2	26.1	24.4	21.6	23.3
TPH + Polar	[g/kg dry soil]	28.3	31.5	30.5	27	24.3	25.9
fluoranthene	[mg/kg dry soil]	4.308	1.696	12.882	3.565	7.16	3.921
benzo(b)fluoranthene	[mg/kg dry soil]	0.866	0.387	3.141	0.54	1.491	0.716
benzo(k)fluoranthene	[mg/kg dry soil]	0.644	0.224	2.107	0.345	1.009	0.464
benzo(a)pyrene	[mg/kg dry soil]	1.397	0.56	4.893	0.844	2.291	1.071
benzo(ghi)perylene	[mg/kg dry soil]	1.01	0.643	2.824	0.742	1.646	0.877
indeno(1,2,3-c,d)pyrene	[mg/kg dry soil]	1.013	0.594	3.877	0.772	1.899	0.938
Cd	[mg/kg dry soil]	1.12	1.78	1.54	1.55	1.5	1.87
Pb	[mg/kg dry soil]	3492	515.5	1265	378.7	335.9	469.6
Zn	[mg/kg dry soil]	636.9	597	590.4	747.8	571.1	657.7
Cu	[mg/kg dry soil]	42.57	81.84	177.2	103.8	121.8	176.7
Ni	[mg/kg dry soil]	24.01	27.53	24.22	22.83	22.1	25.08
Cr	[mg/kg dry soil]	31.98	49.05	32.44	31.43	28.97	31.72
Co	[mg/kg dry soil]	8.37	8.32	8.14	7.71	7.86	7.75
Fe	[mg/kg dry soil]	18607	16903	18713	17148	15907	16157
Mn	[mg/kg dry soil]	364.3	678.6	366.8	373.2	503.3	376.9
Hg	[mg/kg dry soil]	0.24	0.3	0.28	0.49	0.23	0.23
As	[mg/kg dry soil]	9.96	11	7.62	7.67	7.79	7.50

*unamended soil

Table 6. Microbiological analysis of soil at the beginning of bioremediation.

Analysis	Unit	SP 0	1	2	3*	4	5
DAPI bacterial number	(cells/gram)	1.2E+08	2.5E+08	1.2E+08	7.5E+07	2.6E+08	1.4E+08
CW fungal number	(propagules/gram)	3.3E+05	9.9E+05	8.5E+05	6.6E+05	1.5E+06	9.4E+05
Dehydrogenase activity	(TPF/gram)	0	0	0	0.11	0	0

*unamended soil

Bioreactor startup

After soil samples had been taken, manholes of the bioreactor were covered and sealed and aeration of soil began. Following parameters were measured continuously:

- inlet air pressure,
- inlet and outlet air flow,
- inlet and outlet air temperature,
- inlet and outlet air humidity, and
- outlet concentration of CO_2, O_2, and volatile hydrocarbons.

Air flow rate, initially set at 1.1 m^3/hr, was gradually reduced to 0.2 m^3/hr which resulted in decreased oxygen concentration to approximately 15% and increased carbon dioxide to approximately 3.5%. At this point it was decided not to reduce further air flow rate in order to avoid problems with uneven air distribution throughout the bioreactor soil bed. Changes in air flow rate, outlet gas composition, inlet and outlet gas temperature and gas pressure drop are shown in Figure 15 to Figure 18.

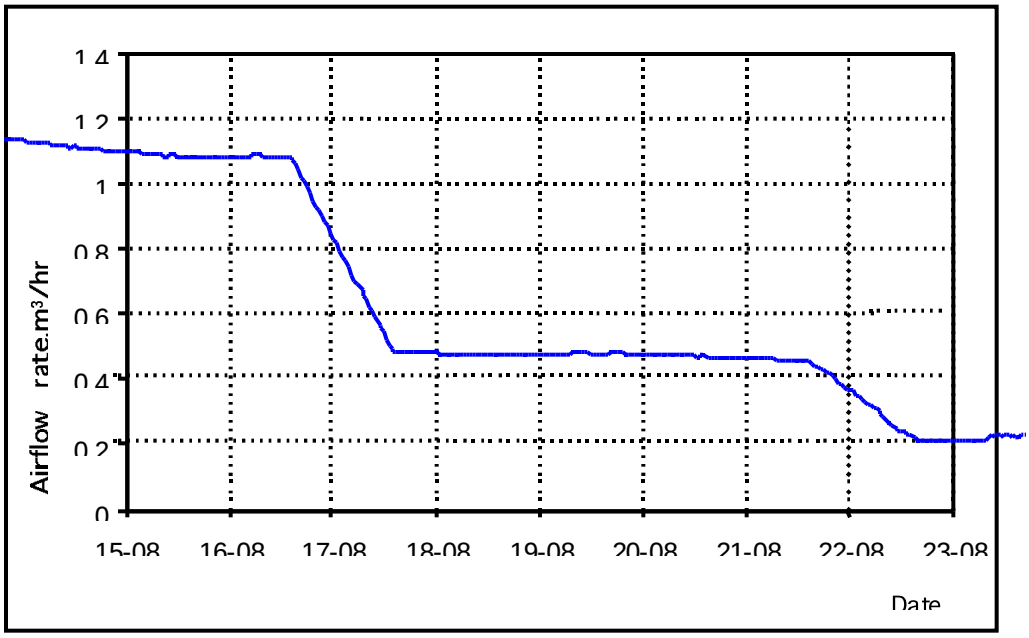


Figure 15. Airflow rate vs. time.

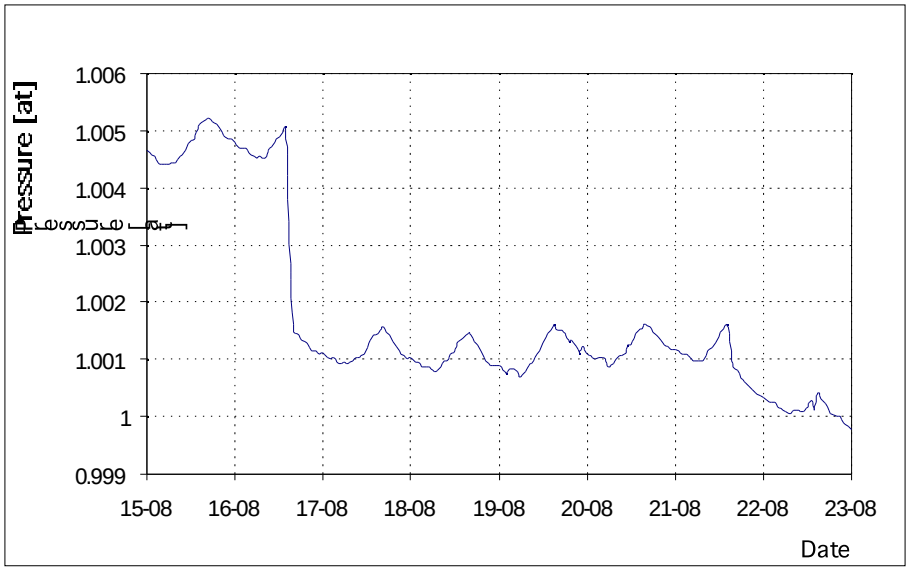


Figure 16. Air flow pressure drop.

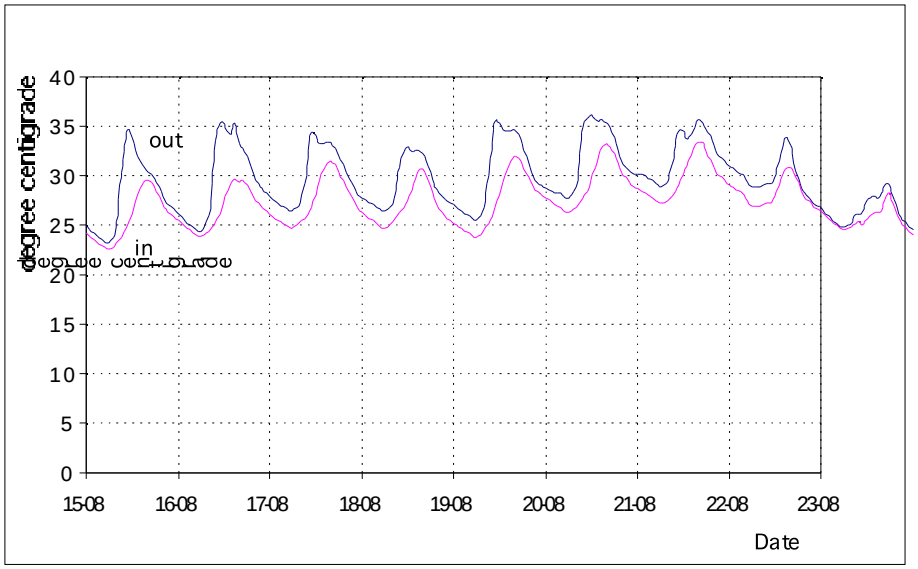


Figure 17. Inlet and outlet air temperatures.

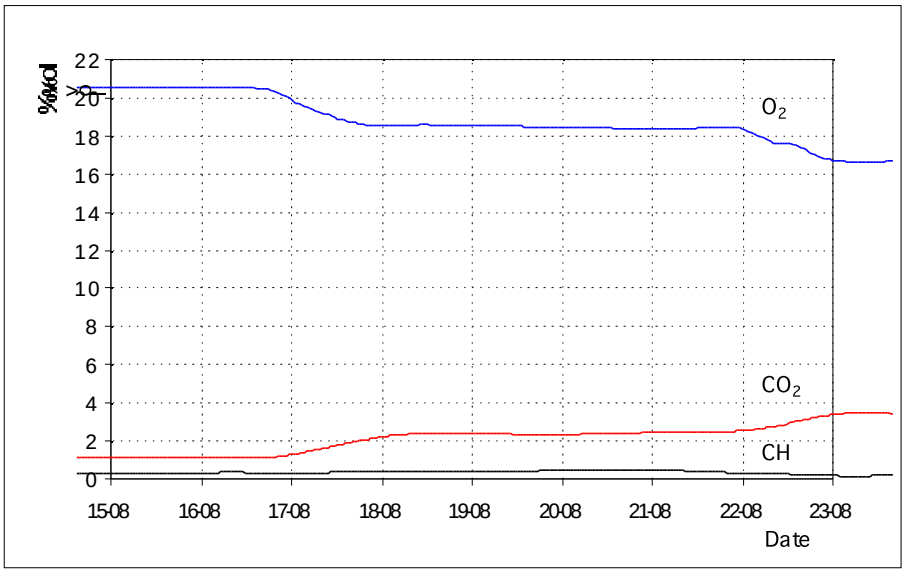


Figure 18. O_2 and CO_2 concentration vs. time.

Bioremediation test conditions

Bioremediation test was carried out at following conditions:

Soil bed geometry: cross area: 6 m^2
depth: 0.4 m
volume: 2.4 m^3

Density of the soil: wood chips mixture was assumed to be ~1,300kg/m^3. Total mass of the remediated soil was ~3.2 metric tons.

Air flow rate: During the first three weeks decreased gradually from 1$m^{3/}$hr to 0.2 $m^{3/}$hr and then was kept constant until the end of the test.

Inlet air temperature: Gradually decreased from 32° C to 17° C. Small diurnal fluctuations caused by differences between day and night temperatures were observed.

Soil bed temperature: changed over the time from 25° C (estimated) at the beginning of the test to ~5° C at the end.

Leachate circulation rate: ~50L/week. During November, leachate was replaced with fresh water in order to decrease concentrations of nutrients.

Soil moisture content: increased gradually over the time of the test from 18.8% to 26%.

Soil gas composition: Oxygen concentration changed from ~20.5% to ~15%vol. depending on air flow rate and intensity of the biodegradation. Carbon dioxide concentration ranged from ~0% to ~4% and its changes showed good consistency with

changes in oxygen concentration. Concentration of volatile hydrocarbons initially were ~0.2% but later dropped below detection limit. Outlet gas was initially saturated with water vapor. When ambient temperature began to drop, gas humidity started to decrease.

Figure 19 to Figure 23 show changes of all parameters measured during bioreactor startup and bioremediation test (between September 1 and October 3 sensors were installed in the bioreactor soil bed).

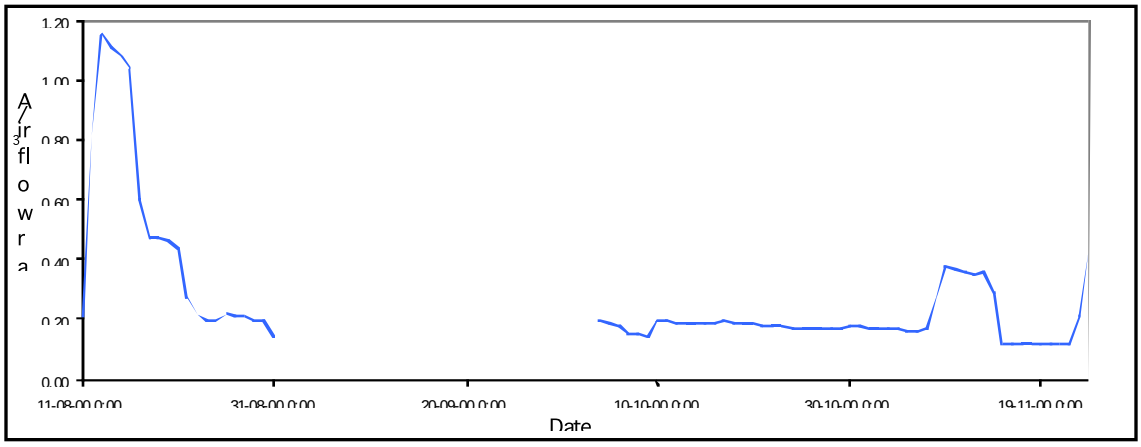


Figure 19. Airflow rate vs. time

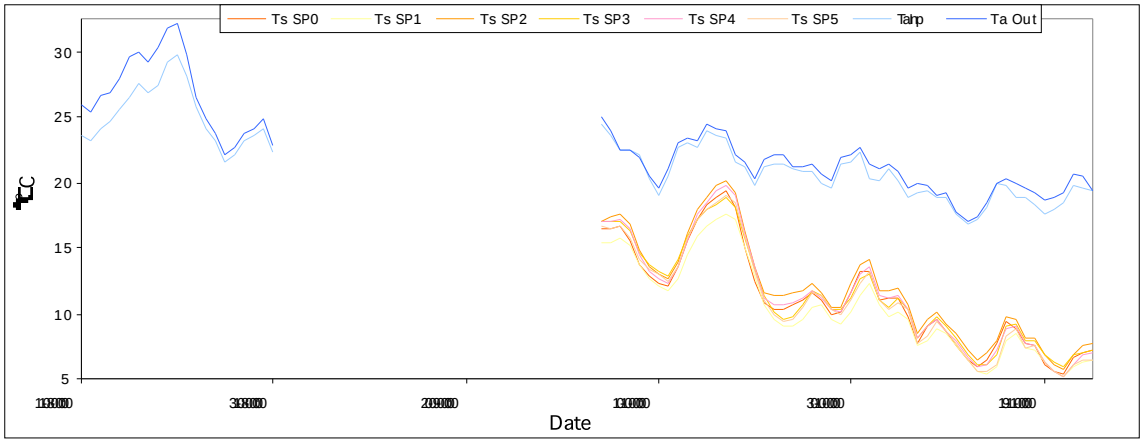


Figure 20. Soil bed and inlet and outlet gases temperature.

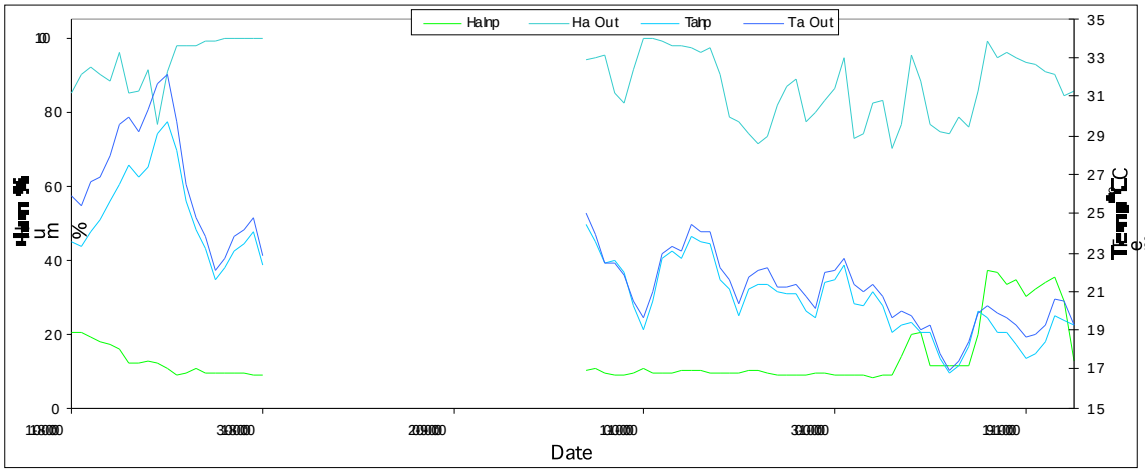


Figure 21. Inlet and outlet gases temperature and humidity.

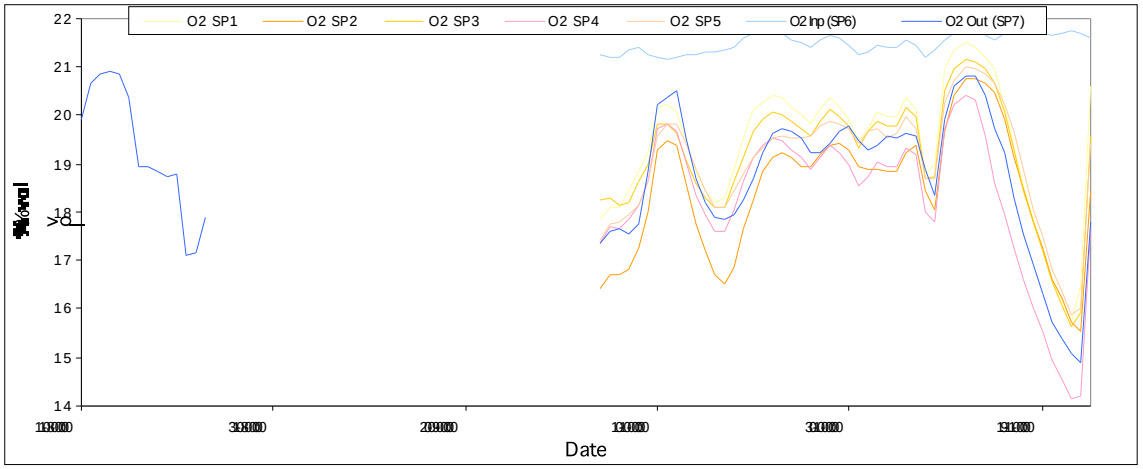


Figure 22. Oxygen concentrations.

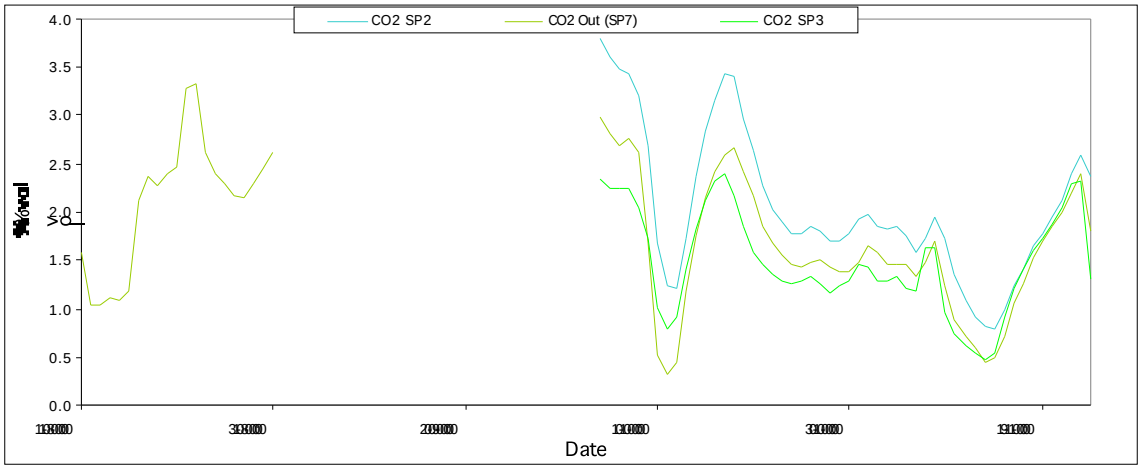


Figure 23. Carbon dioxide concentrations.

Bioremdiation test results

Results of soil chemical and microbiological analyses after three months of bioremediation are shown in Table 7 and Table 8.

Table 7. Chemical analysis of soil after three months of bioremediation.

Analysis	**Unit**	**Sampling Point**					
		SP 0	**SP 1**	**SP 2**	**SP 3***	**SP 4**	**SP 5**
pH in KCl		6.83	6.75	6.81	6.73	6.79	6.98
pH in H_2O		7.14	7.20	7.23	7.13	7.11	7.40
Conductivity	[mS/cm]	5.731	6.586	4.379	2.285	5.842	3907
$N\text{-}NH_4$	[mg/kg dry soil]	5247	6168	4353	2804	5150	5270
$N\text{-}NO_3$	[mg/kg dry soil]	5447	4402	2849	1568	3857	3845
$N\text{-}NO_2$	[mg/kg dry soil]	7.1	3.5	4.2	10.3	1.3	1.4
TKN	[%]	1.34	1.35	1.17	0.69	1.24	1.29
Total P	[%]	0.82	0.81	0.71	0.36	0.84	0.72
PO_4	[mg/kg dry soil]	626	851	43	212	463	520
TPH	[mg/kg dry soil]	14.1	19.7	9.4	15.0	6.9	12.4
TPOC	[mg/kg dry soil]	16.2	23.0	11.4	20.4	9.3	14.6
Fluoranthene	[mg/kg dry soil]	1.603	1.539	1.395	1.060	2.322	1.402
benzo(b)fluoranthene	[mg/kg dry soil]	0.543	0.428	0.279	0.335	0.637	0.331
benzo(k)fluoranthene	[mg/kg dry soil]	0.521	0.276	0.230	0.242	0.488	0.183
benzo(a)pyrene	[mg/kg dry soil]	0.529	0.636	0.545	0.536	0.918	0.448
benzo(ghi)perylene	[mg/kg dry soil]	0.439	0.553	0.450	0.442	0.588	0.388
indeno(1,2,3-c,d)pyrene	[mg/kg dry soil]	0.430	0.426	0.402	0.560	0.878	0.479
Cd.	[mg/kg dry soil]	2.39	2.25	2.38	1.84	2.23	2.17
Pb	[mg/kg dry soil]	1027	600	633	738	511	550
Zn	[mg/kg dry soil]	938	871	1512	767	931	953
Cu	[mg/kg dry soil]	76.0	94.1	81.8	51.6	55.2	101
Ni	[mg/kg dry soil]	24.5	23.7	23.2	25.3	24.2	24.9
Cr	[mg/kg dry soil]	39.7	38.7	36.6	33.5	37.5	36.6
Analysis	**Unit**	**Sampling Point**					

		SP 0	SP 1	SP 2	SP 3*	SP 4	SP 5
Co	[mg/kg dry soil]	8.51	8.24	8.38	8.63	8.45	8.80
Fe	[mg/kg dry soil]	18449	18118	18270	19409	18611	18816
Mn	[mg/kg dry soil]	391.7	409.2	401.6	415.5	413.9	383.1
Hg	[mg/kg dry soil]	0.58	0.47	0.52	0.48	0.46	0.52
As	[mg/kg dry soil]	6.86	6.87	6.65	7.62	7.24	7.68

*unamended soil

Table 8. Microbiological analysis of soil after three months of bioremediation.

Analysis	Unit	SP 0	SP 1	SP 2	SP 3*	SP 4	SP 5
DAPI bacterial #	Cells / g	3.39E+08	3.58E+08	3.25E+08	2.02E+08	4.52E+08	1.32E+08
CW fungal #	Propagules / g	1.51E+06	2.35E+05	2.35E+05	1.41E+05	9.42E+04	6.12E+05
Dehydrogenase activity	TPF / g	0	0	0	20.88	0	0

*unamended soil

TPH content reduction: During the test TPH content in both amended and unamended soil was reduced to ~50% of initial concentration. This result is comparable to results obtained during the column tests in 1999 and much better than in the biopile case. Changes in TPH concentration over time are shown in Figure 24. From this figure and data on nutrient concentrations in amended soil (see Table and Table) it is clear that too much fertilizer was added to soil. As a result, living conditions for the microbial community changed drastically and caused decreased activity. Initially, TPH concentration dropped much more in unamended soil when compared to fertilized soil. However, after microorganisms adapted themselves to new conditions, biodegradation rates in amended soil increased considerably.

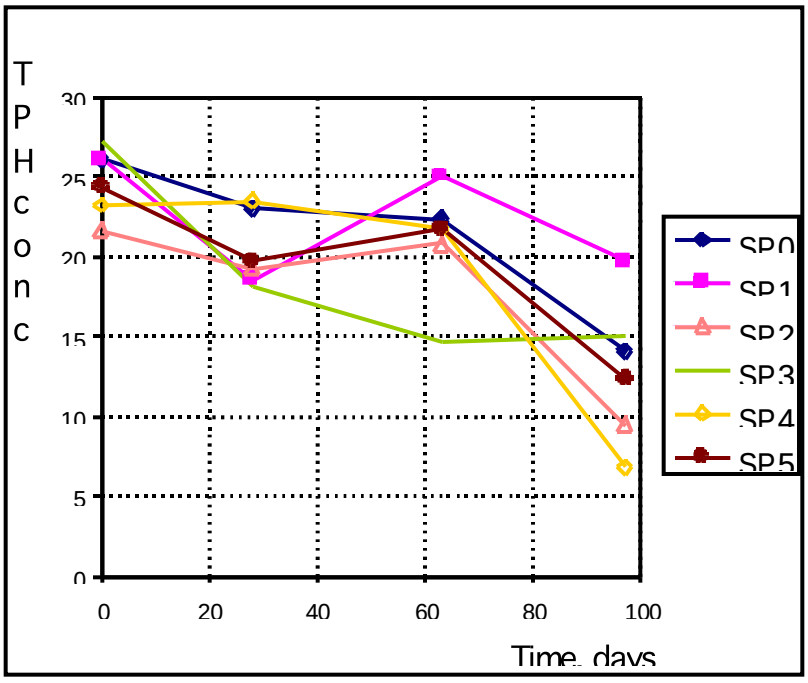


Figure 24. TPH concentration vs. time.

PAH content reduction: Final concentration of PAH in remediated soil was ~ 5mg/kg of soil. On average, reduction in PAH content was ~50% of initial inventory but in SP 2 and SP 3 the reduction attained values of 65% and 80% respectively (see Figure 25).

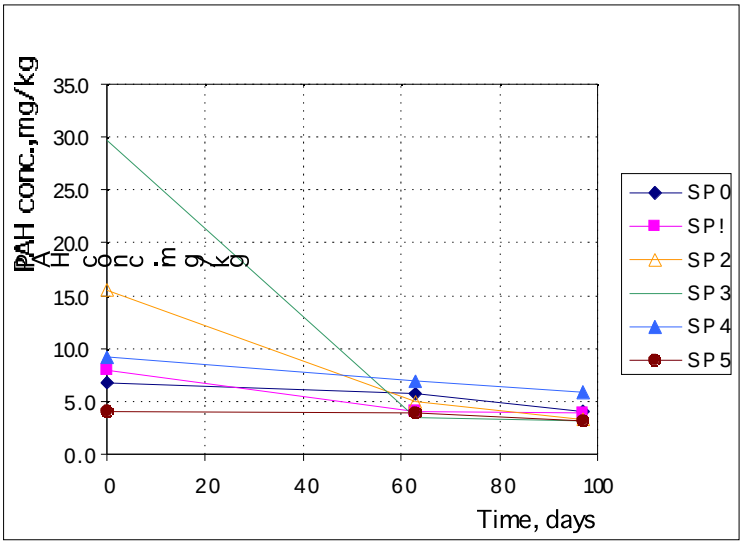


Figure 25. PAH concentrations vs. time.

Bioreactor operation went smoothly. No major technical problems were observed. The test results show that bioreactor construction and data monitoring system combined should provide a useful tool to bioremediate small amounts of petroleum contaminated soil.

Conclusions

1. The main goal of the project was achieved. A 6 m^3 volume, packed bed, mobile bioreactor to remediatelimited amounts of contaminated soils or investigatively derived waste was built and tested.
2. In the test, approximately 3.2 tons of petroleum contaminated soil from Czechowice Oil Refinery, amended with wood chips and fertilizers was treated in the bioreactor for 97 days. During this time TPH and PAH concentrations in soil were reduced to approximately 50% of their initial concentrations.
3. No major problems were encountered with the technical components of the bioreactor.
4. During construction and operation of the bioreactor the following lessons were learned:

a) Mechanical construction:

_ Sealing a regular waste containerwas problematic. If gas tightness of the reactor is important, construction of such a velles “from scratch” would simplify the sealing process.

_ Contaminant degradation rates depend heavily on ambient temperature. Thermal insulation of the bioreactor is postulated in order to limit heat losses and to increase soil bed temperature. Increased bioremediation rates are expected from such modifications.

_ Modifications are planned for the leachate circulation system in order to facilitate its operation. The changes include placing the leachate tank indoors and adding a system enabling for priming the circulation pump when it starts.

b) Bioreactor monitoring system – physical construction

_ The Advantec ADAM modules and the PC based acquisition system turned out to be very useful. Not only did it allow for real-time on-line viewing of sensor output signals as well as corresponding gas concentration, temperature and humidity values, but it also logged collected values into text files. BioReDaq software developed in

IETU is capable of data averaging and logging, controlling sensors power-up and recalculating sensor output signals.

- Gas extraction ports, implemented at each measuring point, were necessary for acquiring gas samples for additional chemical analysis and gas sensors recalibration during bioreactor operation.
- As far as gas flow metering is concerned, bellows gas meters have the best accuracy for observed flow rates.
- During sensor installation in the bioreactor bed, special attention should be paid to avoid privileged flow routes for injected air. The sensors should be wired with plugs built into bioreactor vessels so that they can be easily disassembled.
- Bioreactor controlling and monitoring equipment should be installed out of direct sunlight.

c) Sensors

- As the manufacturer's data are usually averaged for a set of sensors (but not a particular one), sensor characteristics, temperature stability and accuracy should always be determined before installation. This allows individual calibration for each sensor and results in individually improved measurement accuracy.
- Sensor precision and stability may be affected by careless transport and/or operating position. Moreover, physical constraints within the bioreactor may restrict sensor orientaiton. Sensors, which are not sensitive to a single mounting orientation should be used as long as they are economically acceptable.
- Possible interference amongsensors of different types should be considered, (e.g., sensors which generate heat during operation must not be placed near thermocouples or sensors without adequate temperature compensation).
- Sensors and signal devices equipped with temperature compensation should be used whenever possible. Post hoc temperature correction turned out to be difficult and inaccurate, because it is usually impossible to measure temperature at the sensor's active element, and temperature on the sensor's housing is not necessarily the same.
- Sensor characteristics should be checked throughout the bioremediation process, as they tend to change over time. Such calibration can be conducted during bioreactor respiration tests, providing gas sampling ports were installed and another well-calibrated measurement unit is available.

- Oxygen sensors (DRC-XT253) had excellent linear characteristics and were outfitted with good temperature compensation circuits. However, their parameters were different from stated in their technical specification and tend to change over time. They produced low voltage (mV) output signal, so proper wiring was critical to achieve good measurement accuracy. Oxygen sensors required vertical mounting.
- Hydrocarbon sensors (DRC-ADS201) had nonlinear characteristics and they had no temperature compensation. Not only was their calibration process difficult, but, temperature changes had to be considered as well. These sensors produce resistance output signals and require external direct and constant current supply in order to provide signals to the data log system. The hydrocarbon sensors may be mounted in either vertical or horizontal orientation.
- Carbon dioxide sensors (GMM 220) had excellent, linear and time stable characteristics. The output signals were in the low volts range, which made their installation and wiring easy. No calibration procedure was necessary. They did require an external power supply. Because of their large power consumption, and therefore significant heat production, they had to be from the other sensors.
- Thermocouples (DRC-TK024), bellows gas meters (BK-6), pressure sensor (EcoTronic) and humidity sensors (EE-15) worked properly, with good time stability. They did not require calibration procedures.

The results obtained during the test as well as smooth operation showed that the bioreactor construction and the data monitoring system combined could provide a useful tool to biormediate limited amounts of petroleum contaminated soil.

For FY01, it is proposed that DOE and IETU use lessons learned from the CzOR Biopile Project and PCS bioreactor construction and operation. Experience gained in practical soil cleaning and modeling of such processes enable the formulation of criteria and conditions necessary for optimal operation of the PCS bioreactor – a logical next step in developing sound base for use such an apparatus in soil cleaning.

Modifications planned for FY01 include changes to the physical structure (thermal insulation, reconstruction of the leachate circulation system and minor changes in data acquisition system) as well as to the process itself (contaminated soil preparation, loading, and leachate recirculation, as well as the reactor soil bed temperature, moisture

and air flow rate optimization). In addition, the bioreactor will be operated in cooperation with SRTC(the original designers) to accept contaminated water as well as soil. It is proposed that small amounts of contaminated water such as those produced by routine site sampling activities (i.e., investigatively derived waste – IDW) could be remediated using the same system. Both contaminated soil and waste water samples would be taken from the Czechowice Oil Refinery.

6 Appendix A: Sampling and Analysis

6.1 Soil Sampling protocols

Soil samples for analysis were collected using a hand auger and placed in a Whirl-Pak bag or other clean container. Samples were placed in a cooler on ice and managed according to the hold times as seen in Table 9. Prior to sample analysis, samples were weighed to determine the mass of the sample. Laboratory analyses were performed at the IETU laboratory.

6.2 Soil gas sampling protocols

Soil gas composition is measured continuously. Soil gas composition was confirmed periodically using Landtec GEM-500.

6.3 Leachate sampling protocols

Leachate samples from the leachate recirculation system were taken from the leachate tank. Personnel at the IETU laboratory performed the analyses. Analytical methods (organics and inorganics) for leachate samples were analyzed as noted in Table 10.

6.4 Analytical procedures

6.4.1 Chemical Analysis

The analytical procedures listed in Table 10 were used in the analysis of soil leachate samples.

Table 9. Bioreactor Data and Sample Plan

Required Parameters	Soil				Gas					Leachate			Analysis Type	Analyst	Hold Time
	B	D	M	F	B	C	H	M	F	B	W	F			
DAPI	Yes		Yes	Yes						Yes	Yes	Yes	Microbiological	K. Ulfig	3 weeks, 4ºC
CFW (optional)	Yes		Yes	Yes									Microbiological	K. Ulfig	24 hr, 4ºC
Naphtalene-degraders	Yes		Yes	Yes									Microbiological	K. Ulfig	8 hr, 4ºC
Crude Oil-degraders	Yes		Yes	Yes									Microbiological	K. Ulfig	8 hr, 4ºC
Respiration Rates								Yes					Field	M. Adamski	N/A
TTC Activity	Yes		Yes	Yes						Yes	Yes	Yes	Microbiological	K. Ulfig	8 hr, 4ºC
Metals	Yes		Yes	Yes									Lab	T. Manko	Acidified HNO_3
BTEX	Yes		Yes	Yes									Lab	T. Manko	
VOC					Yes			Yes	Yes				Lab	T. Manko	
TPH	Yes		Yes	Yes	Yes			Yes	Yes		Yes		Lab	T. Manko	ASAP, Max 14 d, 4ºC
TPH extract	Yes		Yes	Yes									Lab	T. Manko	Extract<14d & anal<40d
PAH	Yes		Yes	Yes	Yes				Yes				Lab	T. Manko	Extract<14d & anal<40d
% CO_2					Yes		Yes		Yes				Field	M. Adamski	N/A
% CH_4					Yes		Yes		Yes				Field	M. Adamski	N/A
% O_2					Yes		Yes		Yes				Field	M. Adamski	N/A
Moisture	Yes	Yes		Yes									Field	M. Adamski	N/A
$NO_2 + NO_3$	Yes		Yes	Yes						Yes			Lab	T. Manko	ASAP, Max 14 d, 4ºC
NH_4	Yes		Yes	Yes						Yes			Lab	T. Manko	ASAP, Max 14 d, 4ºC
TKN	Yes		Yes	Yes						Yes			Lab	T. Manko	ASAP, Max 14 d, 4ºC
pH	Yes		Yes	Yes						Yes	Yes		Lab	T. Manko	
Conductivity										Yes	Yes		Lab	T. Manko	
PO_4	Yes		Yes	Yes						Yes	Yes		Lab	T. Manko	ASAP or -10ºC
Total P	Yes		Yes	Yes						Yes	Yes		Lab	T. Manko	ASAP or -10ºC
BOD										Yes		Yes	Lab	T. Manko	ASAP, Max 24 hr or -10ºC
COD										Yes		Yes	Lab	T. Manko	ASAP, Max 24 hr or -10ºC

Required Parameters	Soil				Gas					Leachate			Analysis Type	Analyst	Hold Time
	B	D	M	F	B	C	H	M	F	B	W	F			
Temperature	Yes												Field	M. Adamski	N/A
Air Temperature						Yes							Field	M. Adamski	N/A
Air Flow (inlet)						Yes							Field	M. Adamski	N/A
Air Flow (outlet)						Yes							Field	M. Adamski	N/A
B - base, C - continuously, H - hourly, W - weekly, M - monthly, F - final															

Table 10. Methods of Analysis

Soil		
Constituent	**Sample preparation and analytical method**	**Instruments**
BTEX (benzene, toluene, ethylbenzene, total xylenes)	EPA Method 8020	Varian 3400CX chromatograph with SATURN3 GC/MS equipped with SPI injector and capillary column DB-5/MS
PAH (Non-polar Aliphatic Hydrocarbons)	Soil is prepared according to ISO 11464 standard Soil quality – Pretreatment of samples for physico-chemical analysis ISO/DIS 13877: 1998. Soil quality determination of PAH's – method using HPLC.	HPLC chromatograph Series 1050 Hewlett-Packard equipped with a fluorescence detector 1046A Hewlett-Packard and Bakerbond PAH 16-Plus column.
TPH (Total Petroleum Hydrocarbons)	Extraction procedure of soil samples is based on procedure 3520E Extraction method for sludge samples pp 5-28 to 5-29 from Standard Methods for the Examination of Water and Wastewater 18th Edition (1992) with some modifications. PN-V-04007: 1997. Soil protection-tests for petroleum and its component content. Determination of non-polar aliphatic hydrocarbons by IR spectrophotometry.	IR spectrophotometer
Heavy Metals	Soil is prepared according to ISO 11464 standard Soil quality – Pretreatment of samples for physico-chemical analyses. The principle of the method is based on ISO 11466 (Soil quality – extraction of trace elements from soils and related materials by *aqua regia*) with some changes in acid extraction procedure. EPA method 6010B REV. 2 January 1995. Inductively coupled plasma-atomic emission spectroscopy.	AAS and VGA 76 Vapour Generation Accessory (arsenic and mercury) ICP Varian, Liberty 220
Total Kjeldahl Nitrogen	According to ISO 11261	CARY 1 VARIAN
Phosphorus	According to ISO 11263 Soil quality – Spectrometric determination of phosphorus soluble in sodium hydrogen carbonate solution	CARY 1 VARIAN
Nitrite plus Nitrate	According to ISO 11263	CARY 1 VARIAN
Ammonium	According to PN-76/C-04576.01 Water and waste water. Tests for nitrogen; Determination of ammonium nitrogen by colorimetric indophenol method:	CARY 1 VARIAN
Specific conductance	According to ISO 11265 Soil quality – Determination of the specific electrical conductivity.	CX731 conductivity meter (Elmetron, Poland) with a glass cell and temperature compensation probe.

Constituent	Sample preparation and analytical method	Instruments
PH	According ISO 10390 Soil quality - Determination of pH	CX731 pH-meter (Elmetron, Poland) with a combined electrode and temperature compensation probe.
Leachate		
BTEX (benzene, toluene, ethylbenzene, total xylenes)		Same as above
PAH (Non-polar aliphatic Hydrocarbons)	The pH of water samples are neutralized (pH=7) and mixed with 10% (v/v) of isopropanol. The PAHs are extracted, concentrated and cleaned with Baker's SPE column filled with modified silica gel (cyanoNU2 or amino silica gel over C18 silica gel layer) and Baker 12G accessory. PAHs are selectively eluted from SPE comumn with dichloromethane	Same as above
TPH (Total Petroleum Hydrocarbons)		Same as above
Chemical Oxygen Demand (COD)	According to PN-74/C-04578.03	
Heavy metals	The method based on EPA 3005 (TR, TO) and EPA 6010B (ICP)	Same as above
Phosphates	PN-88/C-04537.04: Water and wastewater. Tests for content of phosphorus compounds	CARY 1 VARIAN
Nitrite plus Nitrate	PN-73/C-04576.06 PN-87/C-04576.07	Same as above
Ammonium	PN-76/C-04576.01	Same as above
Specific conductance	EN 27888:1993	Same as above
PH	PN-90/C-04540.01	Same as above

6.4.2 Microbiological Analysis

Microbiological analyses of soil and leachate were performed on a monthly basis. Soil and leachate samples from the bioreactor were collected and processed on the same day the sampling was performed. The DAPI epifluorescence method gives total direct counts of bacterial cells in soil and leachate, using 4,6-Diamindino-2-phenylindole as a fluorochrome. The CFW epifluorescence method gives total direct counts of fungal propagules in soil or leachate, using Calcafluor white as a fluorochrome. The enrichment method provides the Most Probablye Number (MPN) of soil or leachate microorganisms capable of degrading crude oil and naphthalene. The TTC method provides total dehydrogenase activity in soil samples. Microbiological results are recalculated per gram of soil dry weight or per mL of leachate.

6.4.2.1.1 4,6 –Diamindino-2-phenylindole (DAPI) Staining

The DAPI method provides a direct estimate of the total number of bacterial cells in soil and leachate, regardless of ability to grow on any media that might be used. Samples are preserved in phosphate buffered formaldehyde. Soil (1 gram) of leachate (10 mL) is extracted three times with non-ionic homogenizing detergent to remove bacteria from sediment particles. Homogenates are cleared by low speed centrifugationa dn supernatants are pooled. Ten microliters of supernatant is spotted onto each well of a toxoplasmosis microscope slide, stained with 0.5 μg/mL DAPI, rinsed with distilled water, and dried. Numbers of bacterial cells stained with DAPI are counted by epifluorescence microscopy and recalculated per gram of soil dry weight or per mL of leachate. When the bacterial number in a given soil or leachate sample is high, dicimal dilution system in phosphate buffer should be used. Soil dry weight is measured with a weight method (Kepner and Pratt, 1994).

6.4.2.1.2 Calcafluor White (CFW) Staining

The CFW method provides a direct estimate of the total number of fungal propagules in soil or leachate. In this method, the procedure is basically the same as in the DAPI method, except that toxoplasmosis microscope slides are stained with Calcafluor White (Santes et al., 1994).

6.4.2.1.3 Naphthalene and Crude Oil Enrichment

This method provides the Most Probable Number (MPN) of viable microorganisms capable of degrading naphthalene and crude oil in soil and leachate. A phosphate buffer decimal dilution system and Mineral Salt Solution (MSM) are used (Fogel et al., 1986). BIOLOG plates inoculated with soil or leachate dilutions are enriched with naphthalene or crude oil, as sole carbon sources are incubated at 20^{o} and 37^{o} C for three weeks. After incubation, each well is examined for microbial growth on SMA plates (Davies and Evans, 1964). Results are recalculated per gram of soil dry weight or mL of leachate.

6.4.2.1.4 Microbial Dehydrogenase Activity-TTC

Microorganisms employ the electron transport system (ETS) in oxidation of petroleum hydrocarbons. Enzymes of the ETS include a number of dehydrogenases, thus dehydrogenase activity can be used as an overall measure of actvity in soil. Triphenyltetrazolium chloride (TTC) is used as an artificial electron acceptor to estimate

dehydrogenase activity since the reduction of TTC to triphenyl formazan (TPF) causes a color change that can be quantified using a spectrophotometer. Soil samples eare incubated with TTC (1.5 g/100mL) at 37°C for 24 hours. Samples then are extracted with methanol and extracts are measured at 546 nm using a spectrophotometer (Alef and Nannipieri, 1995). Values are given as TPF μg/gdw.

7 Appendix B: Sensor calibration

7.1 Preface

Efficient control of bioreactor performance requires measurements of few gas concentrations in soil as well as inlet and outlet air. Not only must the gas sensors be acquired and tested, but also appropriate calibrating must be done. The process involves determining a mathematical model allowing for gas concentration calculations on sensor output signal basis. It is usually necessary to estimate which factors, other that measured gas concentration,, can affect sensor response. Moreover, functional dependency must be determined so that appropriate corrections can be calculated. Most typical sensor characteristic parameters can be obtained from the manufacturer (or dealer). However, such data are averages for huge amounts of sensors and do not describe the performance of individual sensors.

7.2 Oxygen sensors

Oxygen sensors are intended to measure oxygen concentration in soil gases as well as in outlet and inlet air. All sensors are of the same type, acquired from the same manufacturer. According to the sensor technical specifications, they are equipped with temperature compensation (build inside sensor housing) and their output signal is proportional to the oxygen concentration to which the sensor is exposed. Neither humidity nor other gases influence on sensor output signal was reported. Taking these facts into consideration, a linear model for the sensors was assumed:

$$U=a'c_{02}+b'$$

Where U represents sensor output voltage, c_{02} is oxygen concentration, and a' and b' are model constants. It is more convenient to rewrite the previus equation as follows:

$$C_{02}=aU+b$$

Where *a* and *b* are model constants, which must be estimated during calibration proceses. A two-point calibration method was chosen to determine sensor model constants. This simplification was possible because of linear characteristics. First, signals from the sensors were measured in ambient air. Then sensors were moved into containers with continuous nitrogen flow. After responses from all sensors were established, additional measurements were taken. The measured values, gained for all oxygen sensors, as well as calculated *a* and *b* constants are presented in Table 11.

Table 11. Oxygen sensor responses in air and nitrogen and calculated model constants

Number	**Resp. at 0% O_2**	**Resp. at 20.95% O_2**	**a**	**b**
0	1.41	57.0956	0.3762	-0.5305
1	1.38	58.4922	0.3668.	-0.5062
2	0.86	39.4751	0.5425	-0.4666
3	1.26	51.9828	0.4130	-0.5204
4	1.38	51.8893	1.4148	-0.5724
5	0.55	37.4342	0.5680	-0.3124
6	0.68	43.1134	0.4937	-0.3357
7	1.24	38.7454	0.5586	-0.6926

Sensor responses in ambient air for temperature range of 13.5°C to 26.5°C were observed to determine the accuracy of temperature compensation. Measurement results are presented in Fig. 2. It easily can be seen that temperature changes do not significantly affect sensor output signals and therefore may be neglected.

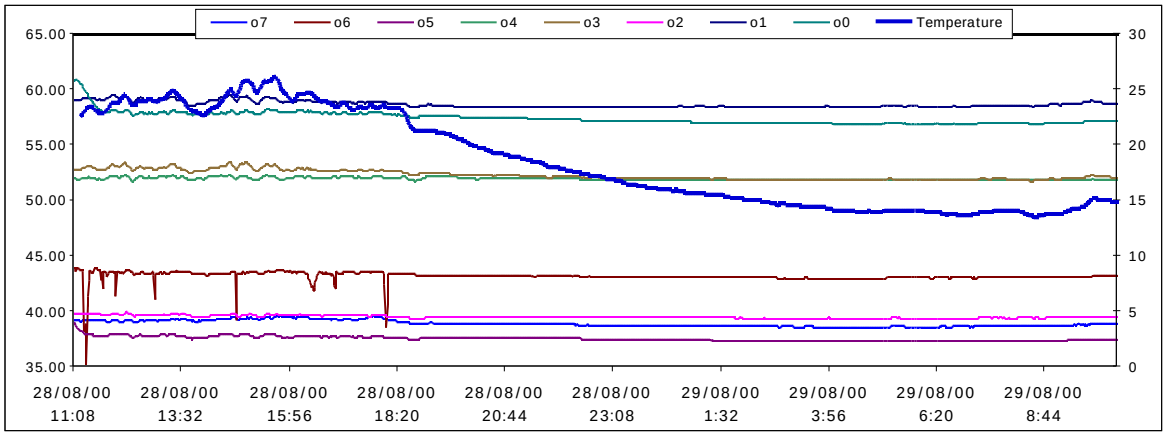


Figure 26. Oxygen sensor responses in air at different temperatures.

7.3 Hydrocarbon sensors

Hydrocarbon sensors, intended for methane concentration measurement in soil gases and inlet/outlet air, are in fact sensitive to a wide range of hydrocarbons. Calibration procedures must be performed in order to measure the specific compound. Calibration for methane includes determining all constants necessary for sensor's model equation so that proper methane concentrations can be calculated, provided the sensor is exposed to gas containing methane, but no other hydrocarbons. All hydrocarbon sensors were acquired from one manyufacturer, and were the same type. Providing constant temperature conditions, dependency between given hydrocarbon concentration and sensor response, according to sensor specification, is yielded by equation:

$$R=R_0 10^{c/k}$$

where R is the sensor's response, R_0 is the sensor's response in clear air, c is measured hydrocarbon (methane in case of the bioreactor) concentration, and k is a model parameter dependent on measured hydrocarbon compounds. As input signals for data acquisition system must be of voltage or current type, a set of constant current sources have been designed and implemented (see previous chapters for details). If each sensor is

supplied by independent, temperature stable, constant current source, then the previous equation may be rewritten more conveniently:

$$U=U_0 10^{c/k}$$

where U and U_0 represent voltage measured on sensor in hydrocarbon polluted and clean air, respectively. By solving for c, the equation may be written as:

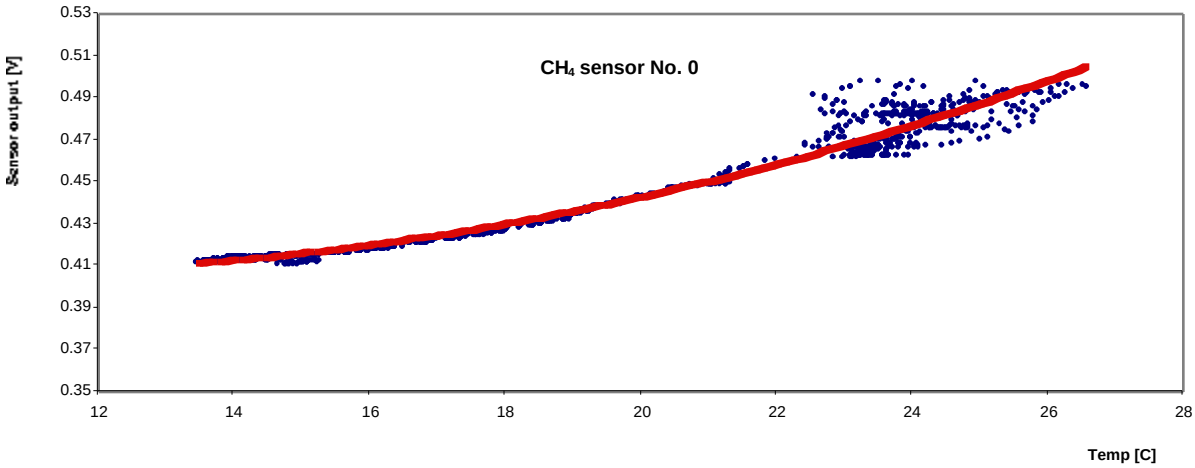
$$C=k\log_{10}(U/U_0)$$

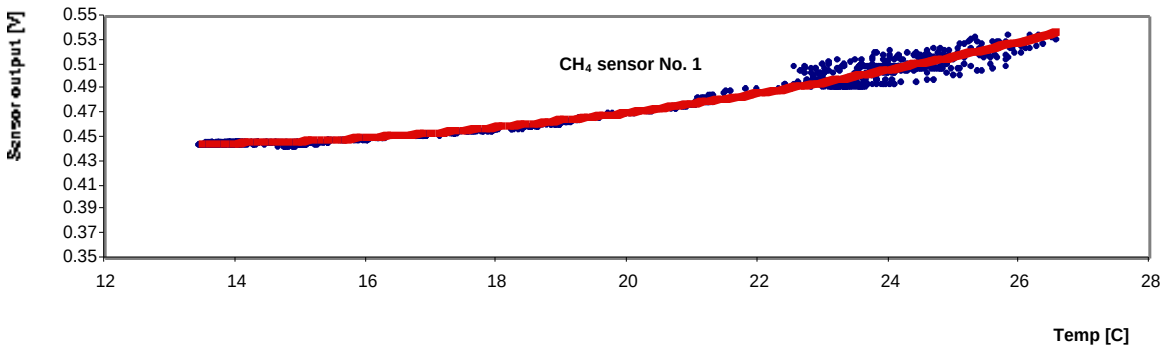
which can be used to obtain hydrocarbon concentration, where sensor output signal is known. Using this relationship requires knowledge of U_0 and k values. Because hydrocarbon sensors have no temperature compensation, both parameters should be considered to be temperature functions: $U_0=U_0(T)$ and $k=k$(T) (see Figure 27). Responses of sensors in clear air were measured at different temperatures (13.5° to 26.5°C) to determine the $U_0=U_0(T)$ dependency. Results are presented in the following plots. The square function was used to approximate U_0 temperature relationship. As U_0 is temperature independent, when the temperature is low enough, the extreme of approximating square functions were calculated and temperature T_m value for that point was chosen as the lowest temperature at which the $U_0=U_0(T)$ relationship cannot be neglected. Values of square function parameters, gained correlation coefficient, constant current source output, as well as T_m values and calculated sensor response at standard temperature (20°C) are presented in Table 12.

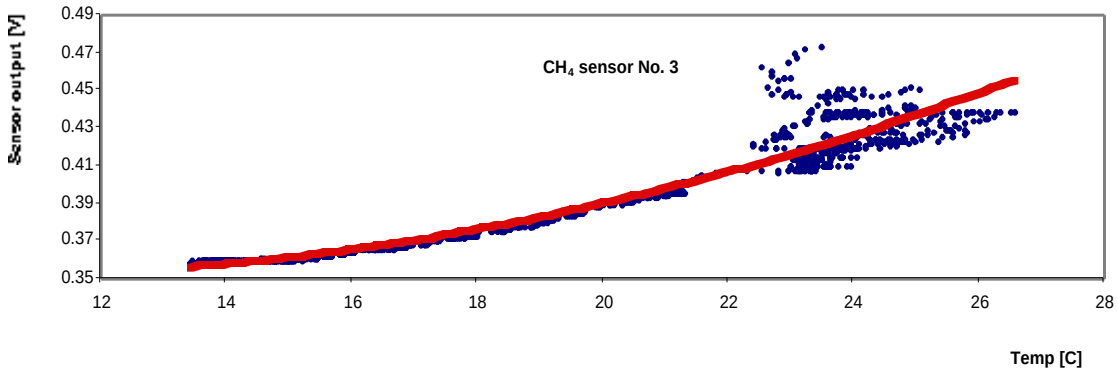
Table 12. Square function parameters, correlation coefficients and T_m, I, U_{Ostd}, R_{Ostd}.

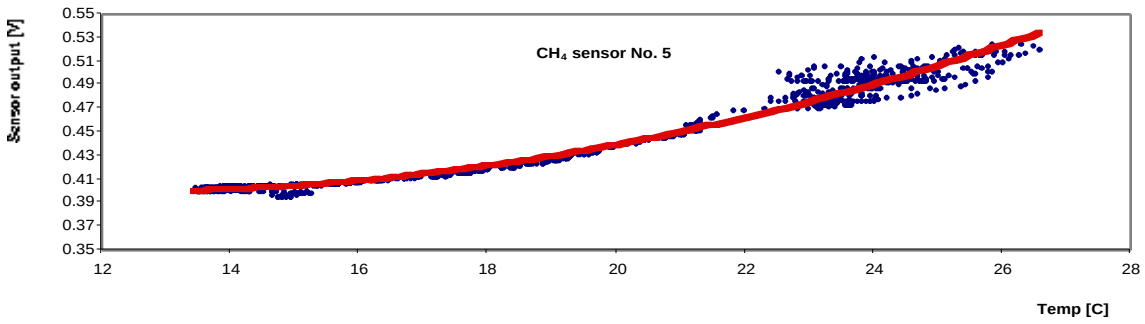
No.	**a**	**b**	**c**	**R**	**T_m [C]**	**U_{Ostd} [V]**	**I [mA]**	**R_{Ostd} [kΩ]**
0	0.0004	-0.0070	0.4417	0.9837	8.75	0.4617	0.585	0.789
1	0.0005	-0.0113	0.5140	0.9889	11.30	0.4880	0.648	0.753
2	0.0006	-0.0168	0.5368	0.9899	14.00	0.4408	0.578	0.763
3	0.0004	-0.0072	0.3862	0.9644	9.00	0.4022	0.587	0.685
4	0.0003	-0.0014	0.3687	0.9945	2.33	0.4607	0.470	0.980
No.	**a**	**b**	**c**	**R**	**T_m [C]**	**U_{Ostd} [V]**	**I [mA]**	**R_{Ostd} [kΩ]**
5	0.0006	-0.0155	0.4923	0.9889	12.92	0.4223	0.516	0.818
6	0.0007	-0.0130	0.4546	0.9957	9.29	0.4746	0.581	0.817
7	0.0003	-0.0050	0.4262	0.9904	8.33	0.4462	0.547	0.816

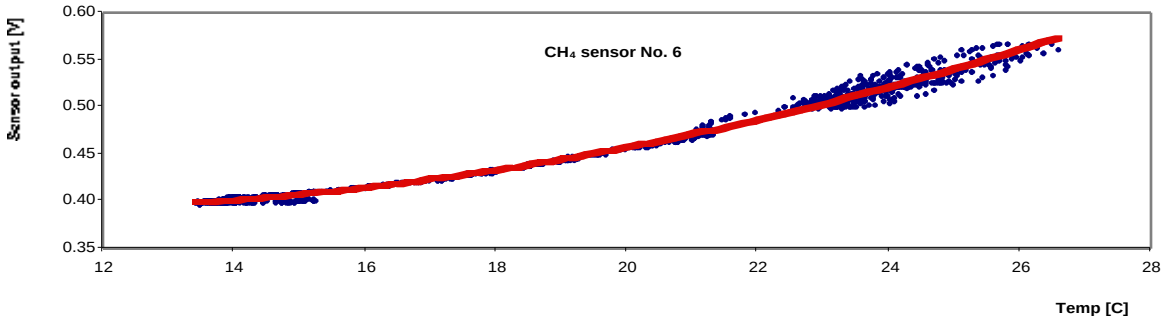
Figure 27. Hydrocarbon sensor responses in clear air as a function of temperature








CH4 sensor No. 5
Sensor output [V]
0.55
0.53
0.51
0.49
0.47
0.45
0.43
0.41
0.39
0.37
0.35
12
14
16
18
20
22
24
26
28
Temp [C]


CH4 sensor No. 6
Sensor output [V]
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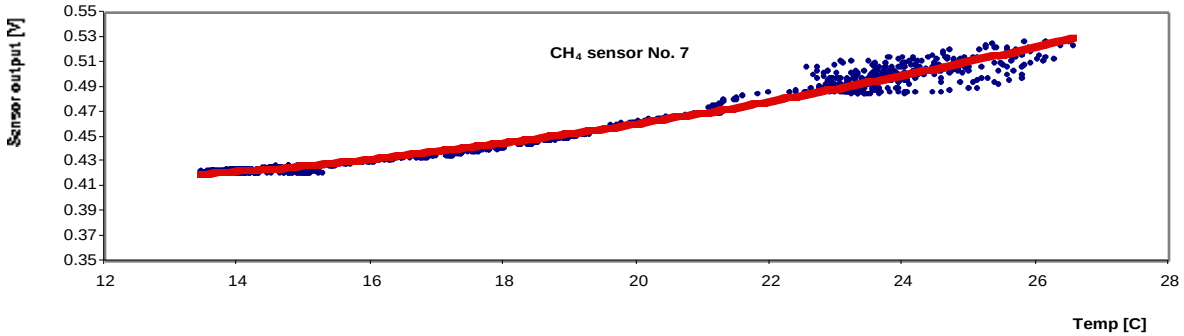
0.55
0.50
0.45
0.40
0.35
12
14
16
18
20
22
24
26
28
Temp [C]


CH4 sensor No. 7
Sensor output [V]
0.55
0.53
0.51
0.49
0.47
0.45
0.43
0.41
0.39
0.37
0.35
12
14
16
18
20
22
24
26
28
Temp [C]

For methane, k=23.75 was assumed to be temperature independent under bioreactor conditions. Its estimation was based on published data (EPA-600-R-92-219 report) and was expected to be the same for each hydrocarbon sensor. Therefore, calculations of methane concentration may be shown as a two step procedure:

1. Recalculating sensor output values measured at known temperature to standard conditions by adding corrections. Correction values are obtained from $U_0=U_0(T)$ dependency.
2. Calculating concentration values from the previously presented model using values of U, U_0 and k known for standard conditions.

The influence of other factors, such as humidity or gas pressure, may be neglected under intended working conditions.

7.4 Carbon dioxide sensor

All carbon dioxide sensors were calibrated and tested by the manufacturer. Output voltage values range from 0 V to 5 V as CO_2 concentration rises from 0% to 20%. Simple, linear relationships are sufficient to recalculate sensor responses to carbon dioxide concentrations. Sensor characteristics and accuracy were determined by comparing responses at different CO_2 concentrations with values measured by means of another measurement device. Measurements were performed during one of the respiration tests so that different levels of carbon dioxide concentration easily could be reached. The sensors were connected to the PC device through ADAM A.D module. Calibrated Geotechnical Instruments GA 94 device was used as an alternative measurement device. All measurements were performed with satisfactory results for each carbon dioxide sensor.

Project Deliverable

Bioremediation Project

FY00 Final Report – Task 3

Bioremediation of Chlorinated Solvents

Submitted to:
U.S. Department of Energy
National Energy Technology Laboratory
Morgantown, West Virginia

Submitted by:
Institute for Ecology of Industrial Areas
Katowice, Poland

Institute for International Cooperative Environmental Research
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Executive Summary

The project entitled “Bioremediation of chlorinated solvents” was conducted as a joint initiative among the Institute for Ecology of Industrial Areas (IETU), the Savannah River Technical Center (SRTC), and Florida State University for the United States Department of Energy (DOE). This review report was prepared based on literature studies concerning biodegradation of chlorinated solvents. The report focuses on the identification of factors and conditions that have been shown to enhance or inhibit the bioremediation of chlorinated solvents. Those findings are summarized in recommendations for follow-up projects. The information on the nature and extent of chlorinated solvent contamination in Poland is based on information from literature and institutional sources. Environmental contamination with chlorinated solvents is still unrecognized in Poland. On a national scale, there is no information on the consumption of this group of chemicals, nor the range of environmental contamination resulting from its release or disposal. We were unable to identify data on the production and use of these chemicals in published statistical surveys. On the local level, such problems are not public information, therefore, access is rather difficult**.**

Due to the sensitive nature of this type of contamination, most private interests with possible contamination were unwilling to allow any type of sample collection. Eventually, a metallurgical site in central Poland agreed to the collection of contaminated soils, but would not agree to any further collaboration.

Soil and groundwater samples from this facility were collected and characterized. The experiments and analytical activities included:

- Sampling of soil and groundwater,
- Development and testing of a method for chemical analysis of chlorinated solvents,
- Performance of chemical analyses, and
- Development and performance of biodegradability tests.

Data collected on the basis of literature studies and results of these tests allowed for preparation of a proposal to apply existing bioremediation expertise to soils contaminated with chlorinated solvents.

Introduction

One of the most common environmental problems in the United States and Western Europe is soil and groundwater contamination with volatile chemical solvents classified as Volatile Organic Compounds (VOCs). In Poland there is no evidence of VOC contamination due to the lack of relevant regulations and site investigations, despite wide use.

Chlorinated aliphatic compounds, notably chlorinated solvents such as tetrachloroethene (PCE) trichloroethene (TCE), trichloroethane (TCA) and carbon tetrachloride (CT) commonly are used as degreasing agents at manufacturing, maintenance and service facilities all over the world. Historically, these compounds have been released into the environment, particularly to soils and groundwater. VOCs are so volatile that they are seldom a problem in surficial soils and surface water *(Anderson, 1995)*. When VOC volatilization from a contaminated matrix is inhibited (e.g., from deep soils or groundwater), they can be persistent because they are relatively stable and resistant to biodegradation. Some of these chlorinated compounds and degradation intermediates may be toxic; some are known or suspected human carcinogens.

Project Objectives

The main objective of the project is to conduct a literature review and initial feasibility studies and, based on the results of those studies, to develop a full proposal to apply existing bioremediation expertise to soils contaminated with chlorinated solvents.

The specific tasks involved in this goal include:

- identifying state-of-the-art chlorinated solvent remediation technologies through a literature review; identifying and reviewing methods that are being applied in Poland and worldwide for remediation of soil contaminated with chlorinated solvents; and analyzing cost-effectiveness and applicability of the methods based on the data from literature and contractors;
- identifying sites in Poland where considerable contamination with chlorinated solvents occurs and where remediation is desirable; choosing a suitable site for this study and for further field-scale investigations and tests; and
- performing chlorinated solvents biodegradability tests under controlled laboratory conditions.

Chlorinated solvents biodegradation – a literature review

Background

Chloroaliphatic compounds with only one or two chlorine substitutes can serve as growth substrates for microorganisms under appropriate conditions. More heavily chlorinated compounds can only be biodegraded by microbes that are provided with an alternate growth substrate (co-metabolism). Bioremediation in the latter case is more complex, but the magnitude of the problem and lack of alternative treatment options have generated a tremendous amount of research on bioremediation of TCE and related solvents. Degradation of the majority of chlorinated solvents occurs by oxidation-reduction reactions that are carried out predominantly by bacteria in the environment. Chlorinated solvent bioremediation involves microorganisms that are naturally present in the subsurface and which can biodegrade solvents to environmentally acceptable products such as carbon dioxide, chloride and water *(Industrial Members, 1997)*. Chlorinated solvents are biodegraded by several processes (Table 1), including:

- direct oxidation, whereby the chlorinated compound is used directly as a microbial growth substrate (food source) and broken down to inorganic molecules such as carbon dioxide, water and chloride,
- reductive dehalogenation, and
- co-metabolism, whereby the chlorinated compound is converted into another chemical by microorganisms during growth on other carbon compounds.

Table 1. List of common chlorinated solvents and their current known degradation pathways.

Degradation Process			**Compound**							
			PCE	TCE	DCE	VC	1,1,1-TCA	CT	CF	DCM
Aerobic biodegradation	As primary substrate		N	N	Y*	Y	N	N	N	Y
	Co-metabolic degradation supported by:	Methane or Alkanes	N	Y	Y	Y	Y*	N	Y	Y
		Aromatic compounds (e. g., toluene)	N	Y	Y	Y	N	N	N	N
		Ammonia	N	Y	Y*	Y*	N	N	Y	Y
Anaerobic biodegradation	As primary substrate		N	N	N	Y	N	N	N	Y
	Co-metabolic degradation under conditions of:	Denitrification	Y*	Y*	Y*	Y*	Y	Y	Y*	Y
		Iron reduction	Y	Y	Y	Y	Y	Y	Y	Y
		Sulfate reduction	Y	Y	Y	Y	Y	Y	Y	Y
		Methanogenesis	Y	Y	Y	Y	Y	Y	Y	Y
Chemical degradation (abiotic transformation)			N	N	N	N	Y	Y*	N	N

Notes:
PCE: Tetrachloroethene (also known as perchloroethene)
TCE: Trichloroethene
DCE: Dichloroethene (statements are true for all isomers)
VC: Vinyl Chloride (also known as chloroethene)
1,1,1-TCA: 1,1,1-Trichloroethane
CT: Carbon Tetrachloride (also known as tetrachloromethane)
CF: Chloroform (also known as trichloromethane)
DCM: Dichloromethane (also known as methylene chloride)
Y: Occurs; concerted opinions in the literature
Y*: May occur: limited evidence or conflicting information
N: Does not occur; concerted opinions in the literature

Natural attenuation

The term “natural attenuation (also known as intrinsic remediation) refers to naturally occurring processes that act without human intervention to reduce the mass, toxicity, mobility, volume or concentration of contaminants in soil and groundwater environments. EPA has estimated that natural attenuation will be effective as the sole remedy at approximately 20% of all chlorinated solvent sites. It also has been estimated that natural attenuation may serve as a portion of the remedy at an additional 50% of all chlorinated solvent sites (Ellis et al., 1996).

Although intrinsic biodegradation is appealing from a number of standpoints, there are some circumstances where it is not appropriate. These include situations where the reductive dechlorination of TCE or PCE does not proceed to completion or proceeds too slowly, and where highly toxic and potent carcinogenic metabolites such as VC or DCE are produced and accumulated. This incomplete metabolism may be due to limitations in electron donors, electron acceptors, or nutrients required to maintain the dechlorinating microbial population or to an absence of appropriate dechlorinating bacteria. In these cases, the potential exists to supply the missing component to initiate or enhance the biodegradation activity. This process is known as accelerated bioremediation, whereby electron donors, electron acceptors, or nutrients are added to the environment to stimulate biological activity (*Harkness et al., 1999*).

Anaerobic degradation

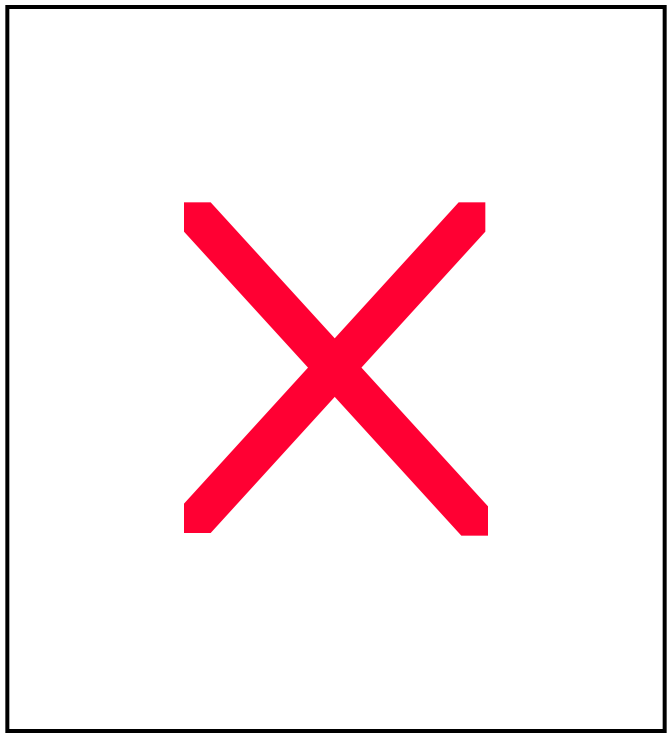
Tetrachloroethane (PCE), a common industrial solvent now widespread in the environment, resists aerobic degradation. Until the early 1980’s PCE and TCE were considered to be persistent in the environment, and to be resistant to microbial degradation (*Infante and Tsongas, 1982*). However, several studies have reported that PCE is transformed via sequential reductive dechlorination under anaerobic conditions both in laboratory experiments (*Bouwer and McCarty, 1983; de Bruin et al., 1992; Di Stefano et al., 1991; Freedman and Gossett, 1989*) and in natural environments (*Parsons et al., 1984*).

The degradation of PCE usually is incomplete, resulting in accumulation of trichloroethene (TCE), dichloroethene (DCE) isomers, and/or vinyl chloride (VC). However, a few studies have reported complete dechlorination to ethene (ETH) (*Di Stefano et al., 1991; Freedman and Gossett, 1989*), whereas *de Bruin et al.,* (1992) found that ethene was further reduced to ethane. Reductive dechlorination of PCE and TCE to ethane or ethene by anaerobic mixed cultures of bacteria has

been demonstrated both in the laboratory and in the field. *Maymo-Gatell et al.* (1997) isolated an organism that is capable of respiratory reductive dechlorination of PCE completely to ETH with H_2 as an electron donor. Because this strain does not appear to belong to any presently known genus or species, its name was suggested as *Dehalococcoides ethenogenes*.

Moreover, these results, coupled with laboratory and field observations have suggested that dechlorination intermediates such as cis-dichloroethene (c-DCE) and vinyl chloride (VC) can be oxidized under aerobic and anaerobic conditions *(Vogel and McCarty, 1985; Bradley and Chapelle, 1996, 1997, 1998; Ferguson and Pietan, 2000*).

Anaerobic transformation pathways (reductions and substitutions) of chlorinated aliphatics, were summarized by Ferguson et al. *(2000)* as follows:



Aerobic degradation and co-metabolism

Chlorinated hydrocarbons are not used as a primary substrate by most heterotrophic organisms, but they are susceptible to aerobic biological degradation by co-metabolism. Co-metabolism results from the expression of nonspecific enzymes that degrade a primary substrate and fortuitously transforms another compound that does not supply carbon, energy, or reducing power to the

organism. The organism requires another compound to supply carbon and energy and in producing enzymes to metabolize the primary substrate, also degrades the target compound.

Currently used auxiliary substrates like methane and toluene have the serious disadvantages of low transformation yields and automatic inactivation due to the formation of highly reactive and toxic metabolites *(Anderson and McCarty, 1997, Little et al., 1988, Wackett and Householder, 1989)*. To avoid automatic inactivation Koziollek et al. *(1998)* supplied chloroethene-degrading bacteria with the use of structural analogues to chloroethenes as the enrichment substrate.

Several studies have revealed that TCE could be transformed aerobically by a consortia of microorganisms (*Fliermans et al., 1988; Fogel et al., 1986*). Co-metabolism of both TCE and DCE by aerobic microorganisms has been studied not only in laboratory scale processes, but also in pilot scale *in situ* bioremediation systems (*Hopkins et al., 1993*). Some soil microorganisms including the ammonia-oxidizing bacteria *Nitrosomonas europaea* (*Arciero et al., 1989*), toluene-oxidizing bacteria (*Nelson et al., 1986*), and cultures of methanotrophic bacteria have been characterized. Methanotrophs are promising bacteria for this purpose *(Yagi et al., 1994)*. They are a group of microorganisms that grow on methane as a sole source of energy and as a major source of carbon (*Little et al., 1988; Fathepure et al., 1988; Tsien et al., 1989; Enzien et al., 1994; Tschantz et al., 1995*). Wilson and Wilson (1985) reported the degradation of TCE in soil columns amended with methane and air. The methane monooxygenase (MMO) systems of methanotrophic bacteria catalyze the incorporation of one oxygen atom from molecular oxygen into methane to produce methanol. These enzymes exist in a soluble or a particulate (membrane-bound) form. The two forms of the enzyme are thought to differ structurally and catalytically. The soluble form of MMO has broader substrate specificity than the membrane-bound (particulate) enzyme. However, the particulate enzyme also oxidizes many compounds other than methane. It has been suggested that the particulate MMO may be responsible for the oxidation of TCE (*Tsien et al., 1989*).

Bioaugmentation

Within the spectrum of bioremediation techniques, bioaugmentation has a specific application. Bioaugmentation involves the introduction of selected exogenous organisms with the desired capabilities directly into the contaminated zones along with any required nutrients to affect the biodegradation of target chemicals. Two distinct bioaugmentation approaches have been developed

for remediating organic compounds. In the first approach, organisms are added to complement or replace the native microbial population. The added microorganisms can be selected for their ability to survive for extended periods or to occupy a specific niche within the contaminated environment. The goal of this approach is to achieve prolonged survival and growth of the added organisms and to degrade target pollutants. In the second bioaugmentation approach, large numbers of bacteria are added to a contaminated environment as biocatalysts that will degrade a significant amount of the target contaminant before becoming inactive or perishing (*Steffan et al., 1999*). The purpose of bioaugmentation is to improve a particular aspect of process performance. In this process a competent consortium or characterized strains, with the ability to degrade the target toxic molecules are used as exogenous inocula for cleaning up polluted sites. Obligate anaerobes could potentially play a useful role of inocula for use in sites contaminated with chlorinated solvents (*DiStefano et al., 1991; Lee et al., 1997; Tokunaga et al., 1998; Fantroussi et al., 1999). Such organisms* can remove halogens from highly halogenated organic compounds by sequential dechlorination. Prior to introducing such microorganisms in the field, effective, reliable, rapid, and relatively inexpensive tools and techniques are required to assess survival and competitiveness of such introduced microbial strains. Several microcosms have been designed to determine the condition sunder which transferred organisms can express their specific activity (*Munakata-Marr et al., 1997; Fantroussi et al., 1997; Harkness et al., 1999; Steffan et al., 1999*). However, these studies have been carried out on a laboratory scale. It appears that one of the challenges for soil bioremediation is "scaling up" these laboratory results to field application and/or *in situ* treatment.

On the basis of the results obtained by Harkness et al. (*1999*), bioaugmentation stimulated complete dechlorination of chlorinated solvents to ethene. This result indicates that bioaugmentation is a viable strategy for remediating chlorinated solvent sites where dechlorination has stopped at or produced c-DCE or VC. Addition of electron donors and nutrients alone stimulate the conversion of TCE to c-DCE, whereas complete dechlorination of c-DCE to ethene was observed only after bioaugmentation. Dechlorination of TCE to c-DCE has been accomplished by accelerated bioremediation using a variety of electron donors, including lactate methanol, butyrate, glutamate and 1,2-propanediol, and toluene, but none of these amendments stimulated c-DCE dechlorination. Neither supplemental nutrients nor longer residence times produced c-DCE dechlorination. Dechlorination of c-DCE to ethene was initiated only after the inoculation of the soil with a competent TCE-dechlorinating bacterial consortium. Results from

this study suggest that accelerated biodegradation and bioaugmentation may represent an effective remedial strategy.

Technological solutions

Remediation of soil and groundwater containing chlorinated solvents has been shown to be costly, time consuming and, in many cases, impractical using standard active technologies (e.g., groundwater extraction and treatment). Bioremediation promises to address these concerns. In the bioremediation of polluted sites, natural, *in situ* processes are favored because they tend to be less expensive. However, in a number of instances, these processes either do not occur, or proceed slowly due to limitations in oxygen (for aerobic bacteria) or reducing agents (for anaerobic bacteria)

Several field demonstrations have been performed using accelerated bioremediation to stimulate the reductive dechlorination of PCE and TCE in groundwater. A field test was carried out by DuPont workers in Victoria, TX, where benzoate and sulfate were used to enhance the reductive dechlorination of PCE (*Beeman et al., 1994*). More recently, benzoate, lactate, and/or methanol have been used to accelerate the dechlorination of PCE and TCE in field tests (*Sewell et al., 1998; Litherland and Anderson, 1997; Spuij et al., 1997; Harkness et al., 1999*).

Indigenous microorganisms are being stimulated to degrade trichloroethene (TCE), tetrachloroethene (PCE) and their daughter products *in situ* by addition of nutrients to the contaminated zone and by surface treatment of the contaminated off-gas and water. The horizontal gaseous nutrient injection wells that form the basis for the DOE Savannah River Site Integrated Demonstration *(Hazen, 1992)* provide significant advantages over conventional *in situ* bioremediation with vertical wells or infiltration galleries. The increased surface area of the horizontal wall supports better delivery of nutrients and easier recovery of gas and water, as well as minimizing clogging and plugging. Methane/air and other gaseous nutrients (nitrous oxide and triethyl phosphate) are injected below the water table. A parallel extraction well was installed and operated in the overlying vadose zone. This strategy encourages nutrient flow and thus biostimulation in the groundwater and vadose zone. Contaminated gases extracted via the vadose zone horizontal extraction well are treated, if necessary, by catalytic oxidation. Catalytic oxidation destroys volatile organic compounds in off-gas leaving only carbon dioxide and HCl.

Beak International Incorporated (BEAK, *1997*) has placed significant emphasis and resources on the development and application of intrinsic and enhanced chlorinated solvents bioremediation in soil and groundwater. Intrinsic bioremediation involves documenting that the rate and extent of natural processes are sufficient to meet site remediation goals or requirements (a.k.a.: natural attenuation). A program of monitoring and process validation must be designed and implemented to ensure the long-term performance and success of this passive remediation alternative.

Enhanced bioremediation involves the addition of nutrients to the subsurface to promote an accelerated rate of solvent bioremediation by indigenous microorganisms present in the subsurface. Nutrients are provided to the microorganisms through engineered nutrient delivery systems that can be active (e.g., flushing/recirculation) or passive (e.g., slow release) depending on site conditions and site remediation goals or requirements. BEAK conducted experiments, along with other geochemical and microbiological characterization studies, to document the complete *in situ* anaerobic dechlorination of tetrachloroethene (PCE) at a chemical transfer facility in Toronto, Ontario. Dechlorination was promoted by methanol, which was a co-contaminant in the groundwater and acetic acid (derived from the acetogenesis of the methanol) as electron donors.

Cost effectiveness

Information on the capital costs related to the application of individual technologies for chlorinated solvents was collected. According to Legrand *(1994)*, there are three biochemical routes to TCE biodegradation: methanotrophic oxidation (MTT), hybrid anaerobic-aerobic process and anaerobic dechlorination. Table 2 presents a preliminary economic evaluation of these three pathways. Each process was defined at full scale and costs were determined. Due to the early stage of development of these technologies, these comparisons of preliminary capital cost of MTT and anaerobic dechlorination are similar but the operating cost of anaerobic dechlorination is much lower.

Table 2. Preliminary economic evaluation of 3 biochemical routes to TCE degradation; groundwater flow = 1,893 L/min, influent = 1 mg TCE/L, effluent < 4.5µg TCE/L (according to *Legrand, 1994*)

Process	**MTT**[1]	**Anaerobic and aerobic process**[2]	**Anaerobic process**[3]
Influent µg TCE/L assumed	1000	1000	1000
µg PCE/L assumed	0	100	100
Capital cost ($ 1000 U.S.)	411	1267	448
Operational cost ($/1000L)	0.21	0.30	0.16
% of operational cost for methane	6%	1%	0%
Electron donor (ethanol)	-	4%	19%
LGAC for effluent polishing	31%	-	-

[1] – Methanotrophic Treatment Technology. Two methanotrophic FBR (fluidized-bed reactor) stages in series based on *Wu et al.* (1992)
[2] - Anaerobic stage followed by methanotrophic (aerobic) stage, based on *Jewell et al.* (1991)
[3]- Single anaerobic stage, based on *de Briun et al*., (1992**)**

Since these capital costs for TCE and PCE bioremediation were estimated only on the basis of pilot and bench scale projects, they are only of informational value. Converting this data to cost per pound for remediation of TCE yields prices between $72 – $136. Such values may be acceptable for laboratory scale, but are not practical for field applications. Unfortunately, no literature information was found on field scale implementation of bioremediation processes for TCE/PCE. However, there are examples in which bioremediation was used together with air stripping, resulting in significant cost reductions. Soil vapor extraction with pump-and-treat technology was compared for the same remediation scenario. Results are presented in Table 3.

Table 3. Cost specification for two cleaning methods applied for soils contaminated with chlorinated solvents

Process	**Air stripping + bioremediation**	**Soil vapor extraction + pump and treat technologies**
Cost per pound of VOC remediated	$21 (depending on bioremediation share in whole process)	$31
Time necessary to complete remediation	4 years	10 years
Total remediation costs	$1 million	$2 million

Not only is the bioremediation-enhanced air stripping less expensive, but it also enables faster site clean up. As stated in the table, the cost per pound of remediated VOC depends on the share of bioremediation in the overall process. If the bioremediation percentage reaches 90% of values gained by air stripping the cost per pound may be as low as $15. Thus, bioremediation and bioremediation enhanced technologies can be more economical than the baseline (pump and treat) technologies. Moreover, bioremediation may be the only technology applicable at sites where it is difficult to extract contaminated groundwater (e.g., sites with clay lenses and/or low permeability sediments).

Soil contamination with chlorinated solvents in Poland

Activities to identify soil and groundwater contamination with chlorinated aliphatic compounds in Poland were carried out in three directions:

- *Institutions dealing with environmental monitoring*. There is no domestic monitoring of soil and water contamination in Poland for chlorinated solvents. According to the National Institute of Hygiene in Warsaw and the Voivodeship Sanitary and Epidemiological Stations, potable water has not been analyzed for the content of chlorinated aliphatic compounds. Exceptions are surface water intake points where the presence of these substances results usually from the application of chloride in water treatment processes.

  According to the National Institute of Hygiene, locally, serious problems were noted related to groundwater contamination with chlorinated aliphatic compounds. However data on the location of these "hot spots" is not available at present.

According to the Environmental Emergency Register run by the Chief Inspector of Environmental Protection in the period of 1996-1999 no accidental releases of chlorinated solvents into the environment have been recorded.

A review of the available literature yielded no additional information.

- *Producers of chlorinated solvents*; The IETU contacted several chemical plants located within the area of Silesian Voivodeship that had produced chlorinated solvents. All of these sites contain hazardous waste disposal sites where soil is known to be contaminated with a wide range of volatile (including chlorinated solvents) and semi-volatile compounds as well as heavy metals.

- *Users of chlorinated solvents;* There is no data on the consumption of these compounds by local industry, However, it is known that in many cases use of these products has been reduced or discontinued. Our contacts with users of chlorinated solvents (i.e., metallurgical plants and dry cleaning facilities) have not resulted in the identification of contaminated soils and/or groundwater except for a single metallurgical plant in central Poland, where site contamination with chlorinated solvents is known. Permission was granted for soil and groundwater sampling at the facility site for the purpose of preliminary investigations.

Summary

Information collected so far allows for the formulation conclusions. Environmental contamination with chlorinated solvents is an unrecognized problem in Poland. National environmental monitoring does not include this group of contaminants. Also, there is no data on their production or application in the published statistical surveys.

Information on local problems with such contaminants is thought to exist, but is not readily available. This situation results from the fact that Polish legal regulations do not refer to any standards on permissible concentrations of chlorinated solvents in surface waters and soil. Activities to establish such standards are ongoing, due to the need for harmonization of Polish regulations with those of the European Union. In the case of soils and groundwater, there are still no effective regulations, but only recommendations. In the table below (Table 4) the recommended allowable concentration of chlorinated solvents in soil and groundwater of Poland are presented.

Table 4. Polish recommended remediation levels
(PIOS, 1995; Guidelines for the assessment of the level of soil and groundwater pollution with petroleum derived contaminants and other chemical substances in remediation processes. Warsaw, Poland, 1995)

Chemical of concern	**Soil**										**Groundwater mg/L**		
	Area A	Area B					Area C				Area		
	A	units for soils [mg/kg s.m.]									A	B	C
		depth [m ∗bgl]											
		0-0.3	0.3-15		>15		0-2	2-15		>15			
		water permeability of soils [m/s]											
			<	>	<	>		<	>				
			1 10^{-7}		1 10^{-7}			1 10^{-7}					
Aliphatic chlorinated single	0.1	0.1	1	5	3	50	60	3	50	*	0.01	10	50
Aliphatic chlorinated total	0.1	0.1	1.5	7	3	70	80	3	70	*	-	15	70

Area A- Agricultural Use
Area B- Multiple Use
Area C- Industrial Use
∗bgl - below ground level

Maximum permissible limits for VOC concentrations are defined only in regulations concerning the quality of potable water. These limits, as specified in the Enclosure No. 1 to the Decree of the Minister of Health and Social Welfare dated 4 May 1990 are presented in Table 5.

Table 5. Requirements for potable water
(Enclosure No 1 to the Decree of the Minister of Health and Social Welfare dated 4 May 1990)

chemical of concern	**unit**	**concentration**
CF	mg/L	0.03
CT	mg/L	0.005
PCE	mg/L	0.01
TCE	mg/L	0.03

Investigations on the possibility of bioremediation of soil contaminated with chlorinated solvents.

Site Conditions

Due to the difficulties described in the previous chapter, no site contaminated with VOCs has been found in which the owners and/or users of which would be interested in carrying out extended field investigations, soil sampling or possible site remediation.

Due to the necessity of conducting preliminary laboratory tests samples were collected from a highly contaminated area of the metallurgical plant in Central Poland; however, the plant is not interested in any further activities on their property.

No data was found on the extent of pollution, geological or hydrological conditions at the site. Site contamination took place in the past. Most of the solvent plume is located under the plant's structures and a hardened surface. No activities to remediate the soil and groundwater have been performed. Periodic sampling is conducted to monitor the spread of the plume.

Sample Collection

Soil and groundwater samples from the metallurgical plant in Central Poland were collected. A geological sampling subcontractor collected the samples. The samples considered to be most heavily contaminated were given to IETU for detailed analyses. Two samples of groundwater and ten samples of soil were obtained, both collected from two soil borings at a depth of up to 7.5 m. Immediately after collection, the samples were placed in 1.5 L glass containers with twist type lids and cooled. They were then transferred to IETU in containers filled with ice for further analyses.

Scope of the chemical analyses

All collected samples were analyzed for VOC content. Samples which, with the highest contamination were selected for microbiological tests and also were analyzed for pH, conductivity, P, N, K, TOC, heavy metals, TPH, PAH, and grain size distribution.

Analytical methods

The analytical procedures listed in Table 6 were used in the analysis of soil and groundwater samples. Samples were delivered to the laboratory for VOC content analysis. Previously, the

IETU has not conducted VOCs analyses. The methodology used for the analysis was developed for this project using the IETU's own financial resources.

Table 6. Methods of Chemical Analysis

Soil		
Constituent	**Sample preparation and analytical method**	**Instruments**
VOC	VOCs was extracted from soil using methanol and determined by means of a Static Head-Space technique / Gas Chromatography / Electron Capture Detection (HS-GC/ECD) ISO/DIS-10301: Water quality - Determination of highly volatile halogenated hydrocarbons - Gas chromatographic method.	Star 3400cx Gas Chromatograph (with DB-624 chromatograph column), combined with Saturn 3 mass spectrophotometer (Varian GC/MS - system) and SPME equipment with Supelco 100 µm PDMS sorption fiber, Varian autosampler with 10 mL volume vials.
PAH (Policyclic Aromatic Hydrocarbons)	Soil is prepared according to ISO 11464 standard Soil quality - Pretreatment of samples for physico-chemical analyses Determination of PAHs in soil is based on ISO/DIS 13877: 1998. Soil quality determination of PAH's – method using HPLC.	HPLC chromatograph Series 1050 Hewlett-Packard equipped with a fluorescence detector 1046A Hewlett-Packard and Bakerbond PAH 16-Plus column
TPH (Total Petroleum Hydrocarbons)	Extraction procedure of soil samples is based on procedure 3620E Extraction method for sludge samples pp 5-28 to 5-29 from Standard Methods for the Examination of Water and Wastewater 18th Edition (1992) with some modifications. EPA Method 8440. Total Recoverable Petroleum Hydrocarbons by Infrared Spectroscopy. Rev. 0. 1995 PN-V-04007: 1997. Soil protection-tests for petroleum and its component content. Determination of non-polar aliphatic hydrocarbons by IR spectrophotometry.	Bruker Vector 22 FT-IR spectrophotometer
Heavy metals	Soil is prepared according to ISO 11464 standard Soil quality - Pretreatment of samples for physico-chemical analyses. The principle of the method is based on ISO 11466 (Soil quality - Extraction of trace elements from soils and related materials by *aqua regia*) with some changes in acid extraction procedure. EPA method 6010B REV. 2. January 1995. Inductively coupled plasma-atomic emission spectroscopy.	AAS and VGA 76 Vapour Generation Accessory (arsenic and mercury) ICP Varian, Liberty 220
Total Kjeldahl Nitrogen	According to ISO 11261	Titrametric method
Phosphorus	Extraction with 1M KCl from field moist soil. Determination according to ISO 11263 Soil quality - Spectrometric determination of phosphorus soluble in sodium hydrogen carbonate solution	CARY 1 VARIAN UV-vis
Nitrite plus Nitrate	Extraction with 1M KCl from field moist soil, determination according to Procedure PB-12 (cadmium column)	CARY 1 VARIAN UV-vis

Soil		
Constituent	**Sample preparation and analytical method**	**Instruments**
Ammonium	According to PN-76/C-04576.01 Water and wastewater. Tests for nitrogen; Determination of ammonium nitrogen by calorimetric indophenol method	CARY 1 VARIAN
Specific conductance	According to ISO 11265 Soil quality - Determination of the specific electrical conductivity.	CX731 conductivity meter (Elmetron, Poland) with a glass cell and temperature compensating probe.
pH	According to ISO 10390 Soil quality - Determination of pH	CX731 pH-meter (Elmetron, Poland) with a combined electrode and a temperature compensating probe.
Groundwater		
Constituent	**Sample preparation and analytical method**	**Instruments**
PAH (Policyclic Aromatic Hydrocarbons)	The pH of water samples are neutralized (pH = 7) and mixed with 10 % (v/v) of isopropanol. The PAHs are extracted, concentrated and cleaned with Baker's SPE column filled with modified silica gel (cyanoNU2 or amino silica gel over C18 silica gel layer) and Baker 12G accessory. PAHs are selectively eluted from SPE column with dichloromethane.	the same as above
TPH (Total Petroleum Hydrocarbons)	The method is based on EPA Method 3620 and PN-82/C-04565.01	the same as above
Heavy metals	The method is based on EPA 3005 (TR, TO) and EPA 6010B (ICP)	the same as above
Phosphates	PN-88/C-04537.04 : Water and wastewater. Tests for content of phosphorus compounds.	CARY 1 VARIAN UV-vis
Nitrite plus Nitrate	PN-73/C-04576.06 PN-87/C-04576.07	the same as above
Ammonium	ISO 7150/1: 1984 Water quality – Determination of ammonium – Part 1: Manual spectrometric method.	the same as above
Specific conductance	EN 27888: 1993	the same as above
pH	PN-90/C-04540.01	the same as above

Microbiological analysis

Microbial activity measurements were conducted using the TTC method (Alef and Nannipieri, 1995). The method is based on the estimation of the TTC (triphenyltetrazolium chloride) reduction rate to TPF (triphenyl formazan) in soils after incubation at 30°C for 24 h.

Field-moist soil (1 g) was weighed into test tubes and mixed with 1 mL of TTC solution. The tubes were incubated for 24 h at 30°C. After the incubation, 20 mL of methanol was added to each tube and tubes were shaken thoroughly. The soil suspension then was filtered. The filters were washed

three times with methanol. The optical density of the clear supernatant was measured against the blank at 546 nm (red color)

Results of the chemical analyses

Data from the analysis of samples in which chlorinated solvent content was identified is presented in Table 7.

Table 7. Data from the analysis of VOCs in soil and groundwater.

Determined VOC	Water µg/L lab # 1129	Water µg/L lab # 1130	Soil mg/kg lab # 1119	Soil mg/kg lab # 1120	Soil mg/kg lab # 1121	Soil mg/kg lab # 1122	Soil mg/kg lab # 1123	Soil mg/kg lab # 1124	Soil mg/kg lab # 1125	Soil mg/kg lab # 1126	Soil mg/kg lab # 1127	Soil mg/kg lab # 1128
Tetrachloroethylene	0.858	24.2	0.249	43.2	7.39	0.959	0.474	0.139	0.00245	0.00276	0.00373	0.00806
Trichloroethylene	551	6880	27.4	609	125	38.2	18.7	84.0	0.0714	0.141	5.49	1.56
cis-1,2-Dichloroethylene	<0.300	<0.300	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006
trans-1,2-Dichloroethylene	<0.510	31.7	<0.010	<0.010	<0.010	<0.010	<0.010	0.261	<0.010	<0.010	<0.010	<0.010
1,1-Dichloroethylene	3.76	<0.380	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Carbon tetrachloride	<0.060	0.188	0.00283	0.016	0.0086	0.0072	0.0044	0.00516	<0.0011	0.0240	0.0138	0.00529
Chloroform	1.47	<0.270	0.0321	0.164	0.0363	<0.0054	<0.0054	<0.0054	0.0237	0.0174	0.0157	0.0137
Methylene chloride	<1.630	<1.630	<0.033	<0.033	<0.033	0.134	<0.033	<0.033	<0.033	0.0845	<0.033	0.0929
1,1,1,2-Tetrachloroethane	<0.065	<0.065	<0.0013	0.529	<0.0013	<0.0013	0.00666	<0.0013	<0.0013	<0.0013	<0.0013	<0.0013
1,1,1-Trichloroethane	14.1	<0.090	0.941	278	2.86	0.832	0.851	0.600	<0.002	<0.002	<0.002	<0.002
1,1,2-Trichloroethane	<0.400	11.4	0.0958	4.85	0.239	0.145	0.107	0.0225	<0.008	<0.008	0.0103	<0.008
1,1-Dichloroethane	11.2	<1.350	<0.030	0.153	<0.030	<0.030	<0.030	<0.030	<0.030	<0.030	<0.030	<0.030
1,2-Dichloroethane	<1.300	<1.300	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025
1,2,3-Trichloropropane	<0.180	<0.180	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035	<0.0035
2,2-Dichloropropane	<0.650	<0.650	<0.013	<0.013	<0.013	<0.013	<0.013	<0.013	<0.013	<0.013	<0.013	<0.013
1,2-Dichloropropane	<0.580	<0.580	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012	<0.012
1,3-Dichloropropane	<1.000	<1.000	<0.020	<0.020	<0.020	<0.020	<0.020	<0.020	0.0604	<0.020	<0.020	<0.020
1,1-Dichloropropene	<0.100	<0.100	<0.002	<0.002	0.0056	<0.002	<0.002	<0.002	0.00500	<0.002	<0.002	<0.002
cis-1,3-Dichloropropene	<0.300	<0.300	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006

Determined VOC	Water µg/L lab # 1129	Water µg/L lab # 1130	Soil mg/kg lab # 1119	Soil mg/kg lab # 1120	Soil mg/kg lab # 1121	Soil mg/kg lab # 1122	Soil mg/kg lab # 1123	Soil mg/kg lab # 1124	Soil mg/kg lab # 1125	Soil mg/kg lab # 1126	Soil mg/kg lab # 1127	Soil mg/kg lab # 1128
trans-1,3-Dichloropropene	<0.410	<0.410	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008	<0.008
Hexachlorobutadiene	<0.010	<0.010	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002
Benzene	<0.200	2.31	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004	<0.004
Chlorobenzene	<0.030	0.130	<0.0005	<0.0005	<0.0005	<0.0005	<0.0005	0.0295	<0.0005	<0.0005	0.0355	0.00333
1,3-Dichlorobenzene	<0.010	<0.010	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	0.0137	<0.0002
1,4-Dichlorobenzene	<0.015	<0.015	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003
1,2,4-Trichlorobenzene	<0.015	<0.015	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003
1,2,3-Trichlorobenzene	<0.015	<0.015	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003
Toluene	0.178	0.396	0.0135	0.436	0.0383	0.0237	0.00915	0.0329	<0.0009	0.00514	0.00744	0.00413
2-Chlorotoluene	<0.010	<0.010	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	0.0182	0.00107
4-Chlorotoluene	<0.010	<0.010	0.00111	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	0.00034	0.0185	0.00169
Ethylbenzene	0.0426	0.167	<0.0003	0.0698	0.0162	0.00541	0.0116	0.00563	0.0128	0.00957	0.00909	0.00258
m-Xylene + p-Xylene	0.509	0.182	0.00493	0.136	0.0082	0.00536	0.00274	0.00606	0.00137	0.00167	0.00205	0.00117
o-Xylene	<0.015	0.0723	0.00259	0.0649	0.00595	0.00106	0.00038	0.00054	0.00232	0.00154	0.00142	0.00100
1,2,4-Trimethylbenzene	0.00314	0.0496	0.00104	0.104	0.00756	0.00185	0.00063	0.00139	0.00081	0.00036	<0.0001	<0.0001
1,3,5-Trimethylbenzene	0.00450	<0.005	<0.0001	0.0187	0.00173	0.00071	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
n-Propylbenzene	<0.010	0.0487	<0.0002	0.192	0.0287	0.00451	0.00330	0.00227	0.00075	<0.0002	<0.0002	<0.0002
Isopropylbenzene	<0.010	<0.010	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
sec-Butylbenzene	<0.005	<0.005	<0.0001	0.0157	0.00382	<0.0001	0.00279	<0.0001	0.00113	0.00062	<0.0001	<0.0001
n-Butylbenzene	<0.005	0.107	<0.0001	0.0421	0.0249	<0.0001	0.00468	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Naphthalene	0.244	<0.010	<0.0002	1.039	0.0334	0.0135	<0.0002	<0.0002	0.0067	<0.0002	<0.0002	<0.0002
Bromoform	<0.270	<0.270	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005

Determined VOC	Water µg/L lab # 1129	Water µg/L lab # 1130	Soil mg/kg lab # 1119	Soil mg/kg lab # 1120	Soil mg/kg lab # 1121	Soil mg/kg lab # 1122	Soil mg/kg lab # 1123	Soil mg/kg lab # 1124	Soil mg/kg lab # 1125	Soil mg/kg lab # 1126	Soil mg/kg lab # 1127	Soil mg/kg lab # 1128
Dibromomethane	<1.270	<1.270	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025
Dibromochloro methane	<0.300	<0.300	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006
1,2-Dibromoethane	<0.550	<0.550	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010	<0.010
Bromodichloro methane	<0.310	<0.310	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006	<0.006
Bromobenzene	<0.028	<0.028	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006	<0.0006

The most heavily contaminated samples were selected for microbiological tests. These soil and groundwater samples (samples no.1130, 1120 and1124) were analyzed for pH, conductivity, P, N, K, TOC, heavy metals, TPH, PAH and grain size analysis. Data from the analyses is presented in tables 8, 9, 10, 11 and 12.

Table 8. Data from the analysis of soil for TPH, TPOC and PAH.

Sample Parameter	Units	Soil (1120)	Soil (1124)
TPH	[g/kg dry soil]	10.1	23.5
TOC	[g/kg dry soil]	10.4	22.2
Fluoranthene	[mg/kg]	0.361	0.623
Benzo(b)fluoranthene	[mg/kg]	0.091	0.087
Benzo(k)fluoranthene	[mg/kg]	0.041	0.077
Benzo(a)pyrene	[mg/kg]	0.018	0.143
Benzo(ghi)perylene	[mg/kg]	0.051	0.142
indeno(1,2,3-c,d)pyrene	[mg/kg]	0.076	0.104

Table 9. Soil data for metals, conductivity, pH, TOC, P (total) and nitrogen compounds

Sample Parameter	Units	Soil (1120)	Soil (1124)
pH H_20		8.22	6.85
pH KCl		6.46	6.05
Conductivity	µS/cm	314	136
N - NH_4	mg/kg dry soil	5.91	4.66
N – NO_3	mg/kg dry soil	0.226	0.113
N – NO_2	mg/kg dry soil	<0.13	<0.10
TKN	(%)	0.029	0.082
TOC	(%)	1.60	3.30
P total	mg/kg dry soil	<0.25	<0.25
Cd	mg/kg dry soil	0.05	0.57
Pb	mg/kg dry soil	19.19	34.30
Zn	mg/kg dry soil	56.87	392.0
Cu	mg/kg dry soil	36.91	35.65
Ni	mg/kg dry soil	22.45	3.96
Cr	mg/kg dry soil	15.78	9.56
Fe	mg/kg dry soil	7252	6173
Mn	mg/kg dry soil	185.6	303.4
Hg	mg/kg dry soil	0.28	0.80
As	mg/kg dry soil	3.01	3.03

Table 10. Groundwater data for metals, conductivity, pH and nitrogen compounds

Sample		Water 1130
Parameter	**Unit**	
pH		8.78
conductivity	µS/cm	568
N- NH_4	mg/L	0.048
N - NO_3	mg/L	<0.02
N - NO_2	mg/L	<0.02
Cd	µg/L	0.732
Pb	µg/L	8.17
Zn	µg/L	22.3
Cu	µg/L	37.9
Ni	µg/L	21.2
Cr	µg/L	1.54
Fe	µg/L	643
Mn	µg/L	3617
Hg	µg/L	0.43

Table 11. TPH, TPOC and PAH groundwater results

Sample		Groundwater 1130	
Parameter	**Unit**	**Water** *	**Oil**
TPH	mg/L	295	308
TPOC	mg/L	299	335
fluoranthene	ug/L	1.642	
benzo(b)fluoranthene	µg/L	0.274	
benzo(k)fluoranthene	µg/L	0.085	
benzo(a)pyrene	µg/L	0.232	
benzo(ghi)perylene	µg/L	0.312	
indeno(1,2,3-c,d)pyrene	µg/L	0.177	

* Water sample contained oily free-product that was analyzed separately.

Table 12. Grain size analysis of soil

Sample **Grain size composition, fractions**		**Soil 1120**	**Soil 1124**
> 1 mm	%	27.0	25.4
1-0.5mm	%	17.9	16.5
0.5-0.25 mm	%	30.3	44.2
0.25-0.125 mm	%	18.3	11.0
0.0125-0.071 mm	%	4.0	1.6
< 0.071 mm	%	2.5	1.3

Results of the microbiological analyses

Microbial investigation showed that the soil samples (in fact a sandy soil) were poor in terms of biological activity. Organic matter and nutrient analyses found very low levels of each. As such the soils are not conducive to microbiological processes. Microbial activity of these samples, as measured by the TTC method, was zero. For subsequent microbiological experiments, sewage sludge was used as a source of organic matter and microorganisms.

Biodegradation tests

The objective of these experiments was to determine the potential for and rates of biodegradation (biodechlorination) of TCE and its daughter compounds in soil under anaerobic conditions. Experimental conditions that would optimize anaerobic biodechlorination of TCE were established based on literature reviews. Anaerobic mixed-bacterial consortia were obtained from anaerobically digested sludge sampled at one of the Upper Silesian wastewater treatment plants. Two soil samples with high levels of TCE-contamination were selected for examination in the experiment.

Description of the experiment:

In the experiment, the methods of Lee *et al.* (1997) and Tokunaga *et al.* (1998) were used. Also, some anaerobic microbial strains were isolated for further experiments.

To determine the potential for TCE biodegradation, 5 g of contaminated soil and 1 g of anaerobic sludge as an inoculum were placed in 125 mL serum bottles filled with 50 mL of minimal broth (MB) medium each. The composition of MB was as follows (per 1 liter of demineralized water):

Component	Quantity	Purpose
Potassium dihydrogen phosphate	7 grams	Media components to grow and maintain cell cultures
Dipotassium hydrogen phosphate	2.0 grams	
Magnesium sulfate	0.1grams	
Sodium citrate 2-hydrate	1.0 grams	
Yeast extract	1.5 grams	
Trace mineral solution	1.0 mL	
Resazurin	4.0 mg	Redox indicator

Subsequently, the headspaces of bottles were flushed with deoxygenated N_2. Then the bottles were sealed with a Teflon-coated rubber and aluminum septum-cap. TCE was added to the bottles using a microsyringe to obtain final concentration of about 117 mg/L. Incubation proceeded at 25°C for 2 weeks. Six replicates of each combination of the experiment, (i.e., blank, contaminated soil, contaminated soil + anaerobic sludge) were prepared. At the beginning and at the end of the experiment, concentrations of TCE and its derivatives, chloride ion concentrations, and pH of the medium were determined. An Orion meter and electrode was calibrated at pH 4 and 7 using Orion application buffer solutions and was used to measure pH. During incubation, the redox indicator, resazurin dye, changed its color as a function of pH. Resazurin was colorless when the potential was < -200 mV and became pink as the potentials increased (-100 to -120 mV).

The test bottles were opened after headspace analyses, and pH and microbial counts in the liquid phase were analyzed. After a 2-week incubation, a new experiment designed to isolate TCE dechlorinating microorganisms was started.

Isolation of TCE dechlorinating microorganisms from anaerobic cultures

The purpose of the experiment was to isolate and purify microbial strains growing under anaerobic conditions. Cultures were grown under anaerobic conditions at 25°C for 21 days. During the incubation the bacteria was continuously exposed to TCE vapors. This was accomplished placing cotton, saturated with TCE into a microtube. The microtube was then taped to the inside of the covered culture plate. Isolates were obtained by plating serial dilutions on MB (minimal broth) medium.

Bacterial growth was observed on the plates where soil + anaerobic sewage sludge was added to the medium (Picture 3) (Appendix). Microbial degradation of TCE was observed in the same samples.

In total, 50 colonies were randomly collected from three replicate plates of each treatment. Isolates were taken from the plates and inoculated on SMA (Standard Method Agar - Difco) to obtain pure, simple colonies. The purity of the isolates was confirmed by plating on SMA. Colonies will be

further characterized in terms of cell morphology, identification such as Gram reaction and API test (bioMerieux).

VOC determination:

Trichloroethylene and its biodegradation products in the experimental samples containing MB with soil and sewage sludge were determined by means of a Static HeadSpace technique / Gas Chromatography / Electron Capture Detection (HS-GC/ECD).

A 50 mL sample of nutrient water solution with (or without) soil or sewage sludge (used in different combination depending on the experiment treatment), was placed in a 125 mL glass vial and hermetically capped with a Teflon-coated rubber and aluminum septum-cap. Air in the capped vial was replaced with nitrogen by streaming nitrogen through the bottles (20m/min). The nitrogen stream was conveyed by a needle inserted into the plug (gases escaped through a second, similar needle also inserted into the plug). As a result, approximately 500 mL of nitrogen was forced through each vial in order to remove the air. Nitrogen flow was measured with a rotameter. Following the nitrogen purge, 4 µL of trichloroethene was injected through the plug into each vial and the mixture was hand-shaken. Standards were prepared in a similar manner except that the nutrients were in the solution (no soil or sewage sludge). Half of the above mentioned samples and standard solutions were exposed to thermodynamic stabilization process carried out at ambient temperature for 24 hours. The contents were analyzed for TCE and its derivatives by means of the HS/GC/ECD technique. The other half of the sample replicates was put into a shaker and was incubated at ambient temperature for 14 days. Gas from the head space (10 µL) was taken by means of a gas-tight syringe and then introduced manually to the gas chromatographic batcher. The separation and quantitative analysis were made using a capillary gas chromatography and electron capture detector (ECD). The most difficult part of the method was optimization of the sample split in the chromatographic batcher because of the need to simultaneously determine high concentrations of TCE and trace quantities of its decomposition products by means of the ECD detector. At high TCE concentrations the ECD shows non-linearity which complicates this process.

Chloride ion$^-$ determination

Analyses of chloride ions by means of an ion chromatograph equipped with a standard column proved to be impossible due to disturbances caused by high concentrations of phosphate and

sulfate ions introduced from the nutrients. Therefore the traditional, but less precise method was used the so called "opacity / turbidity" method.

Results of microbiological tests

The course of the microbiological test was illustrated in Appendix figures 1 and 2. The results of chemical analyses carried out at the beginning and at the end of the test are presented in tables 13, 14 and 15.

Table 13. pH changes during the experiment

	Blank sample	**Soil 1 (no. 1120)**	**Soil 2 (no. 1124)**	**Soil 1 + sludge**	**Soil 2 + sludge**
Start	6.06	6.12	6.10	6.08	6.10
End	6.00	6.04	6.30	6.12	6.25

Table 14. VOC content in samples before and after the experiment

Contaminant	**Start**					**End**				
(μg/sample)	**B**	**S1**	**S1+Slu.**	**S2**	**S2+Slu.**	**B**	**S1**	**S1+Slu.**	**S2**	**S2+Slu.**
TCE	5710	8376	10550	4960	5454	5625	7744	7954	4704	5147
cis-1,2-DCE	ND	ND	ND	ND	ND	ND	184.5	115.3	157.7	124
1,1-DCE	ND	ND	ND	ND	ND	ND	3.09	3.15	4.26	3.21
VC	ND	ND	ND	ND	ND	ND	ND	25	3.73	328

ND - not determined
B - blank sample
Slu. - sludge
S - soil

Table 15. Concentration of chloride ions in samples (after filtration) before and after the experiment

Contaminant (mg/L)	Start					End				
	B	S1	S1+Slu.	S2	S2+Slu.	B	S1	S1+Slu.	S2	S2+Slu.
Cl^-	3.23	5.14	6.95	5.19	8.73	4.21	5.64	9.06	3.98	6.55

Discussion of the chemical analysis and microbiological test results

Chemical analysis

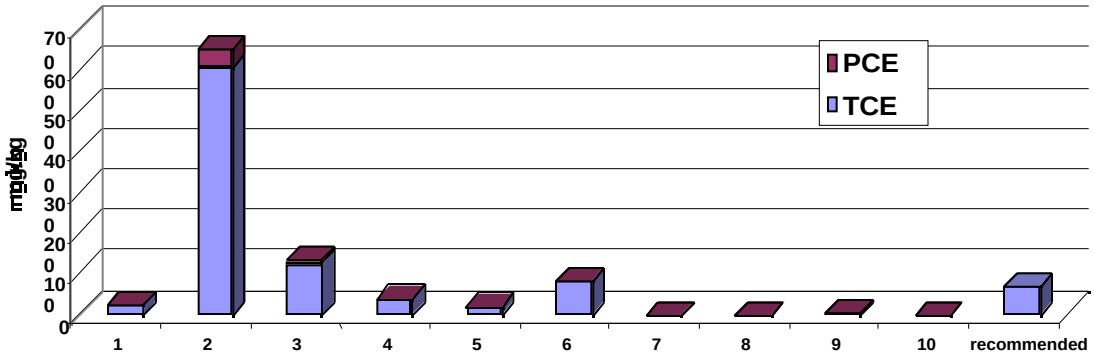
Both soil and groundwater samples showed significant PCE and TCE contamination (Table 7). Three samples of soil and both samples of groundwater show concentrations of VOCs that were above regulatory recommendations (Figure 1). The more detailed analyses of soil samples most heavily contaminated with VOCs consist of a sandy soil with a majority of fractions greater than 0.25 mm (Table 12), and characterized by low levels of organic carbon and nutrients (Table 9). Such soil is not conducive to microbiological processes. This was confirmed by the results of the initial experiments:

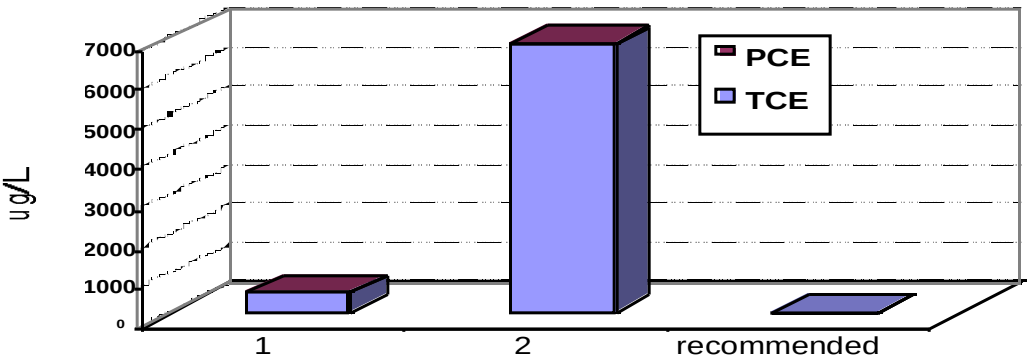
- no significant generation of TCE and PCE degradation products, (DCE and VC) indicating a lack of spontaneous biodegradation processes, and
- no microbiological activity in the analyzed soil.

Concentrations of other pollutants (tables 8 and 9) are not expected to hinder the degradation of VOC's:

- concentrations of heavy metals are below the permissible limits for agricultural soils,
- presence of petroleum-derived contaminants (TPH) should support
- bioremediation processes by providing additional energy sources for bacteria.

Figure. 1. Chlorinated solvent concentration in soil and groundwater samples from the metallurgical plant in Central Poland and Polish recommended remediation level (PIOS, 1995)





Microbiological tests

While the results of these initial bioremediation tests are promising, they should be treated as preliminary. The relatively small reduction in TCE concentration (Table 16) can be attributed to the short time span of the experiment. The depletion of up to 25% of the TCE content obtained in one of the cases (similar in 3 replications) was not reflected in corresponding increases in DCE and VC. The increase of chloride ions observed in this case is not sufficient to prove dechlorination of TCE due to the low precision of the analytical method. Moreover, it seems that relatively high contents of chloride ions, both in the comparative sample as well as in samples analyzed at the beginning of the experiment, eliminate the possibility of considering chloride ion increase as a measure of dechlorination progress control, even if a more precise analytical method was applied.

Table16. TCE content in samples (% of initial mass) after the experiment

Contaminant	End				
	B	S1	S1+Slu.	S2	S2+Slu.
TCE [% of initial content]	98.5	92.4	75.39	94.8	94.7

Despite the limitations in interpretation of the results, it can be concluded that the experiment confirmed the possibility of bioremediating soils contaminated with VOCs under anaerobic conditions.

Anaerobic reductive dechlorination supports the growth of novel, specialized microorganisms that use organic chlorine as electron acceptor. In these conditions, complete transformation is not usually seen. Biotransformation of TCE observed in these experiments was the result of the activity of mixed microbial communities, which were obtained from anaerobic sludge. Sewage sludge is a heterogenous mixture, which is very rich in organic matter and microorganisms, and is high in microbiological activity.

These results lead to the following conclusions:

- TCE was biodegraded under anaerobic conditions to isomeric DCE (a relatively safe metabolite) and vinyl chloride (a recalcitrant carcinogen).
- Incomplete reductive dechlorination of TCE under anaerobic conditions was observed in the experiment (resulting in accumulation of DCE isomers and VC).
- TCE dechlorination in contaminated soils by natural microbial communities is slow compared to the anaerobic sludge amended samples. The addition of the sewage sludge to the soil enhances biodegradation of TCE and improves conditions of the cultures.

Work Plan for FY01

Goal

The goal of the FY01 studies is to develop a technology for bioremediation of chlorinated solvents using a bioreactor.

Research material

Research will be carried out using artificially contaminated soil. The soil type and contamination level will be selected to model actual site conditions found in the United States.

Description of the bioremediation method selected for the research

(on the basis of information collected from literature studies and experiments)

The literature studies show that there are many different methods for bioremediation of soil and groundwater contaminated with VOCs. Initially, the most attractive method seemed to be the anaerobic dechlorination method due to its low costs and relatively simple application. However, preliminary laboratory tests have found low reaction rates and accumulation of VC, which is mutagenic and toxic. In order to move quickly to address the field problem, it has been decided to adopt the well known and tested (including work by SRTC) method of methanotrophic oxidation of TCE. A hybrid anaerobic and aerobic biodegradation mechanism supplemented with methanol as an electron donor and conducted in soil columns (Enzien et al., 1994) did not produce toxic dechlorination products (DCE and VC).

Laboratory studies

Laboratory studies will be carried out (in 125 mL serum bottles) to assess the impacts of various waste substances as electron donors and the addition of aerobic and anaerobic sewage sludge as inoculum on the bioremediation process of VOC contaminated soil (methane biostimulation method). Additional tests will be carried out to determine the TCE biodegradation capacity of the previously isolated bacterial strains. The impact of bacterial strains isolated in FY00 experiments also will be checked.

Experiments in the bioreactor

The new bioreactor will be identical in dimensions to the bioreactor constructed for the FY00 project (bioremediation of petroleum-contaminated soils). One of the most important aspects will be to make sure that the new bioreactor is air tight, so that the whole process (both aerobic and anaerobic) can proceed in the same vessel. The bioreactor will be equipped with a special gas mixture supply system, which will supply gas mixtures of various composition and a gas composition control system to control the composition of gas carried away from the bioreactor with a degassing option. The gas supply system will allow the bioreactor to work both in aerobic and anaerobic conditions.

A detailed research plan and a brief technical design for the construction of the bioreactor will be presented in the FY01 Test Plan.

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Project Deliverable

Phytoremediation Project

FY00 Final Report – Task 4

Cost - Effective Phytoremediation

Submitted to:

U.S. Department of Energy

National Energy Technology Laboratory

Morgantown, West Virginia

Submitted by:

Institute for Ecology of Industrial Areas

Katowice, Poland

Institute for International Cooperative Environmental Research

Florida State University

Tallahassee, Florida, USA

November 2000

Executive Summary

In the process of phytoextraction, amendments, primarily chelating agents, facilitate plant uptake of metals from contaminated soil, and also account for the majority of phytoremediation costs. Previously, the quantity of amendments to be used was calculated based on information of local soil properties, plant species and pollutants concentrations.

The original approach taken in this project used standard agricultural spray equipment and resulted in a wide distribution of amendments to both the plants and soil. The cost of amendments was found to represent ~ 70% of the total cost. As such, loss of soil amendments to overspray increased costs. In addition, plants respond negatively when exposed to soil amendments. In order to reduce damage to the crop and cost of the process, a second generation field-deployed soil amendment dispenser was designed and built. The dispenser applies amendments on the soil, close to the plant stems. As a result material waste and the detrimental effects of soil amendments to plant leaves are reduced. The device was designed to move easily through the field and not to damage plants during application. The device worked well and provided a potential opportunity for savings in amendment application.

In the case of anthropogenic soil pollution, the pollutants are typically distributed unevenly over the site. A system that automatically controls the distribution of amendments could apply amendments in relation to the actual concentrations of metals in soil resulting in significant savings. In order to attain this objective, a third generation amendment dispenser was designed and built. This control device utilizes data from a "computer map" of pollutant concentration at the site to modulate amendment application.

Prototype testing progressed from laboratory to field. Initial field tests were conducted using a test application pattern on bare ground in order to visually confirm the operation of the system. System testing culminated with successful amendment application to a field planted with *Brassica*.

It was estimated that during the course of the project, the major cost factor (i.e., soil amendments) was reduced by as much as 50%, resulting in overall savings of 35%. FY01 plans call for a full-scale deployment of the current generation dispenser system. Cost and performance data will be collected to document the operational parameters of this device.

Introduction

The aim of the F2000 Project is to develop advanced technologies for the application of amendments to contaminated soils in support of phytoremdiation. As indicated in the 1998 Final report, as much as 70% of the total costs of the phytoremediation process is spent on amendments. Therefore, this year's investigations were focused on optimizing the application of amendments to soils and improving the accuracy of amendment application. A second focus was to design a system that would allow interaction between the degree of soil pollution and the intensity of amendment application. It is anticipated that the potential decrease in cost associated with these modifications is approximately 30% of the total cost of large scale phytoremediation.

Addressing the objectives identified above required the integration of GIS, computer software/hardware and machinery design experts. Once project details were accepted by FSU/DOE, subcontractor were chosen and work commenced. The test site for development of this technology was the field used for the 1999 project. Field characterization data from that project were used as a test case for this project. A timeline was developed to ensure that each step would be completed in time to meet overall project schedules (see figures 1 and 2).

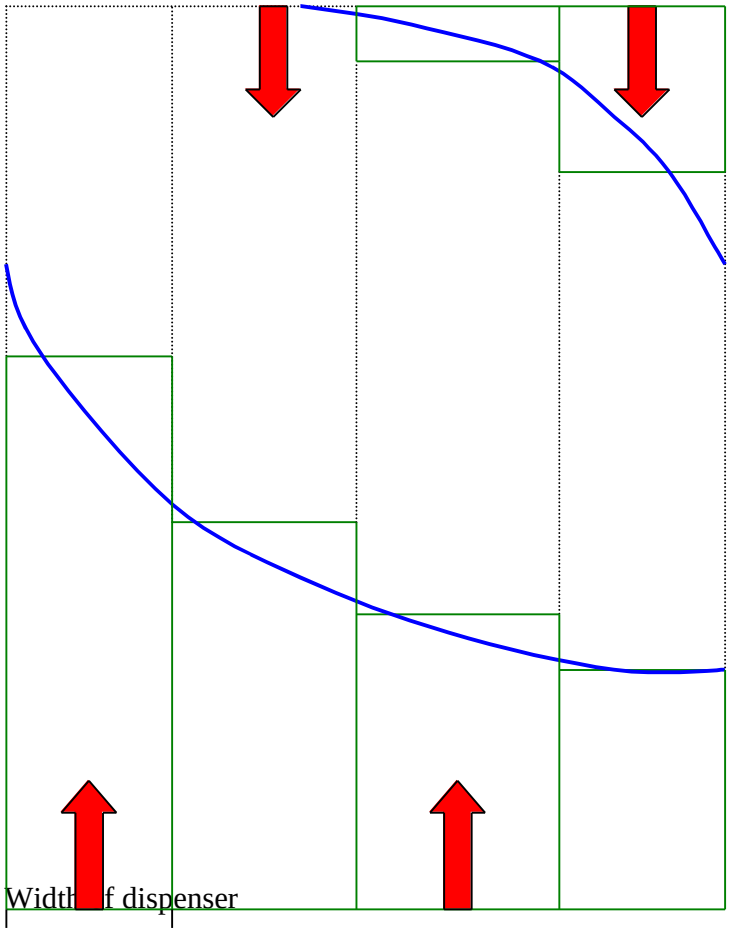

Figure 1. Distribution of amendment application areas for the CF2 field. Areas outlined in green are those which were above the target level of 500 mg/kg lead in soil. Amendments were applied only applied to those areas.

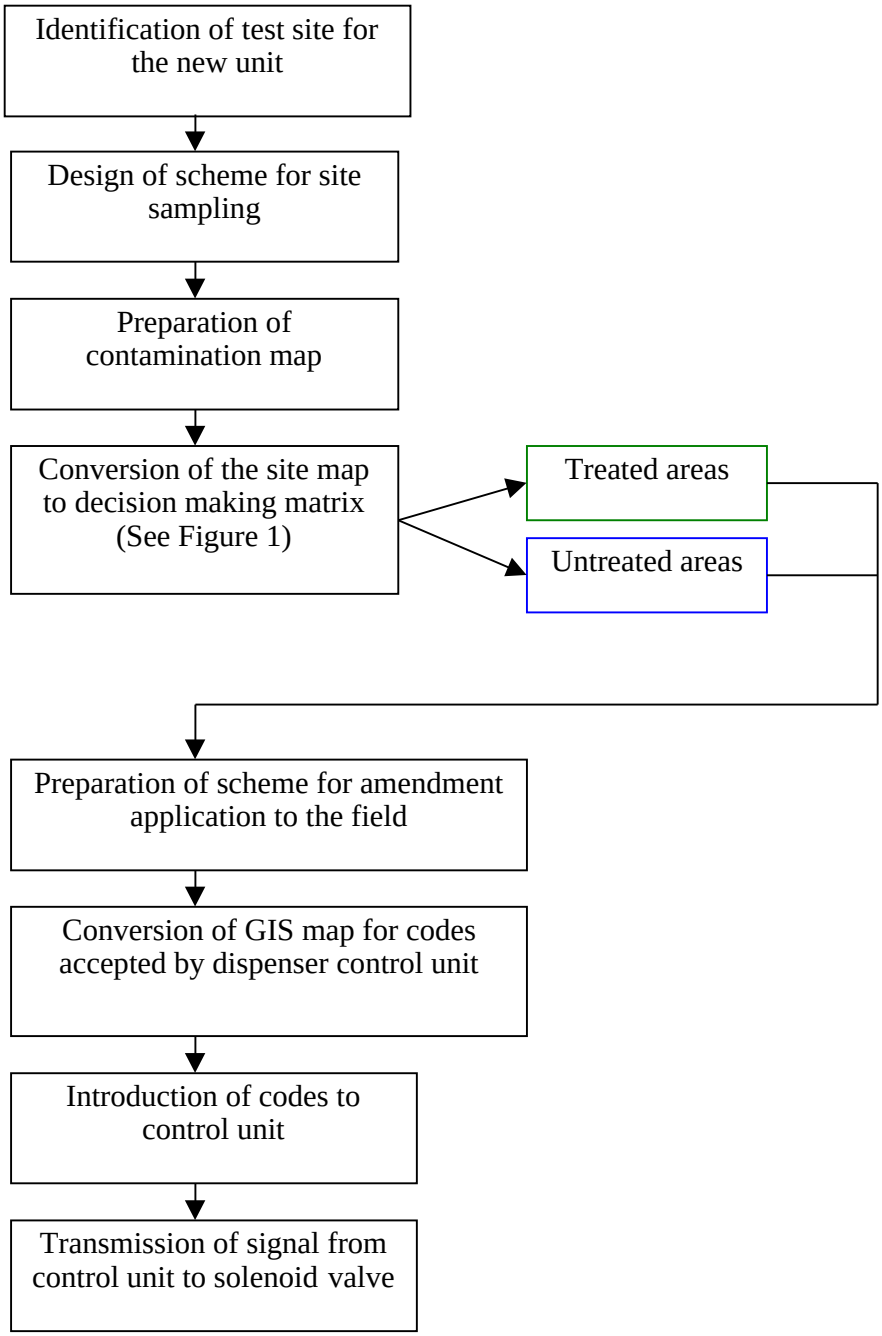

Figure 2. Design process schematic for amendment dispersion controlling unit.

Amendment Application Controller Design Principals

The development of equipment to control the application of amendments to contaminated soil in support of the phytoextraction process was divided into the following steps:

- Manufacturing of electronic components including software and PC hardware. (Figure 3).

- Production of mechanical subassemblies (a distance measuring wheel to provide location information) and execution subassemblies (an assembly of electromagnetic valves to meter amendment application). (Figure 4).

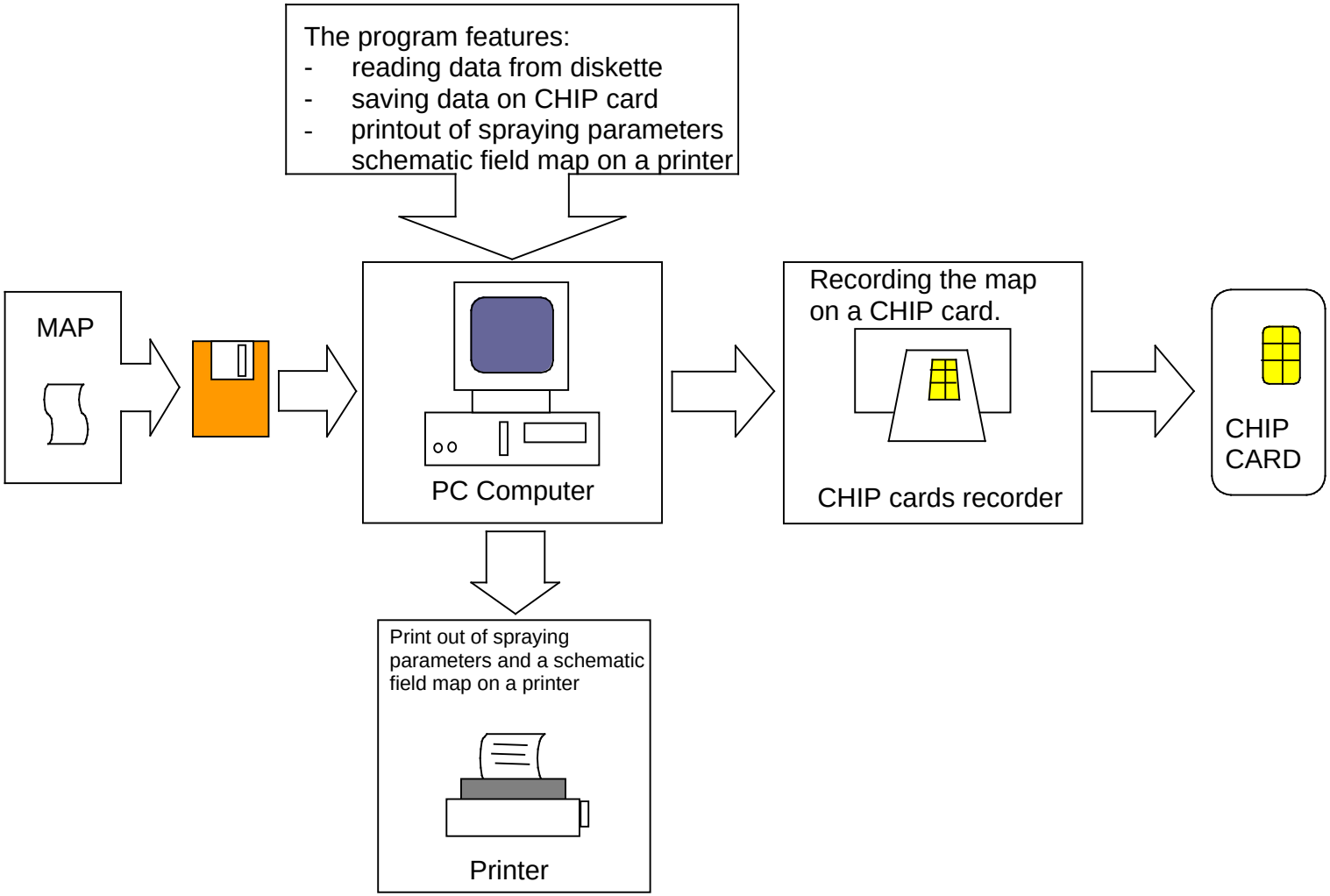

Figure 3. Scheme of electronic components of control unit.

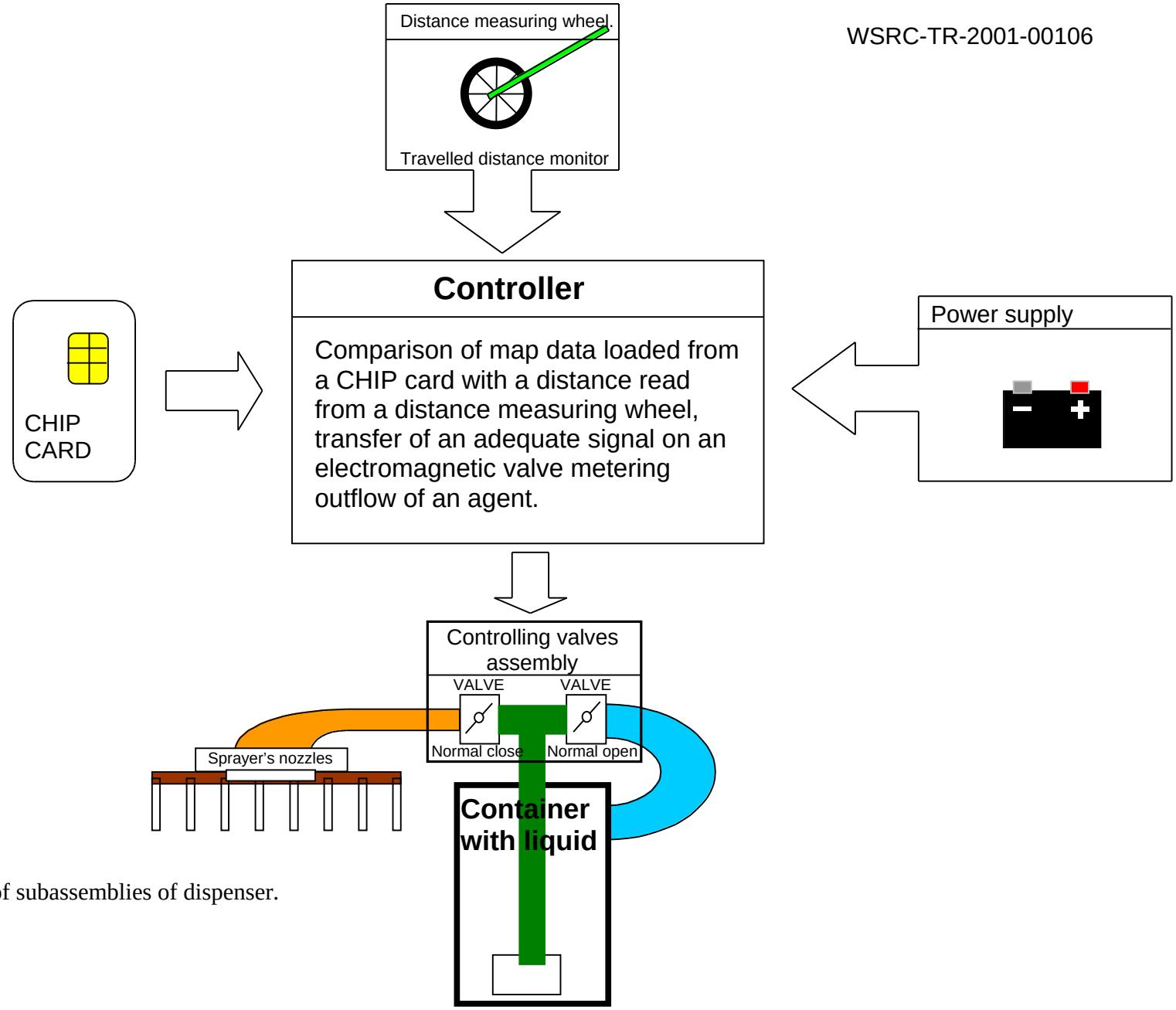

Figure 4. Scheme of subassemblies of dispenser.

The electronic-computer system includes:

- a program that inputs lead concentration values for the site from a diskette to computer memory;
- a computer program that saves this data on a CHIP card;
- a computer program that outputs application parameters and a schematic map of the target field; and
- an electronic controller that outputs execution signals (apply/do not apply amendment instructions).

The electronic controller consists of:

- CHIP card recorder
- electronic-computer system which:

  a) compares map data loaded from a CHIP card with distance read from a distance measuring wheel,

  b) transmits appropriate signals to the electromagnetic valve assembly metering liquid outflow,
- a control panel allowing the operator to monitor equipment operation via an LCD display,
- connections for the independent power supply, signal input leads from the distance measuring wheel and output signals that control the execution valve assembly.

Tests of controller

Three test steps were carried out during the project:

Laboratory tests
Preliminary (parking lot) tests
Field tests (crop 1 and crop 2).

The laboratory tests of the computer-driven control unit were conducted successfully. ASCII-transformed GIS data from the experimental site were downloaded from a PC to the control unit using a magnetic CHIP card. The main functions of the control unit were tested. The unit successfully monitored distance traveled, opened and closed the distribution valves appropriately and correctly calculated and displayed distance traveled, distance remaining and liquid consumption.

Figure 5. Laboratory tests of dispenser

The control unit was combined with the amendment dispenser apparatus, installed on a tractor and tested. Preliminary tests were performed on a parking lot at the Cooperative Farm (CF). The field test of the dispenser was performed at the CF2 field that had been planted previously with Indian mustard (*Brasica juncea*). In order to conduct the field test, a digital map of soil contamination was prepared. This map used a lead concentration in soil of 300 mg/kg as the critical value for determining whether or not to apply amendments. The following table provides an example of the data set transmitted to the amendment control computer.

Table 1. Amendment application by row.

Row #	Row Length (m)	Apply (+)/Do not apply (-) distance (m)
1	110	+110
2	110	+110
3	110	+110
4	110	+110
5	110	+110
6	110	+110
7	110	+10, -20, +80
8	110	+80, -20, +10
9	110	-60, +50
10	110	+50, -60
11	110	+40, -30, +40
12	110	+40, -30, +40
13	110	+40, -30, +40
14	110	+40, -30, +40
15	110	-70, +40

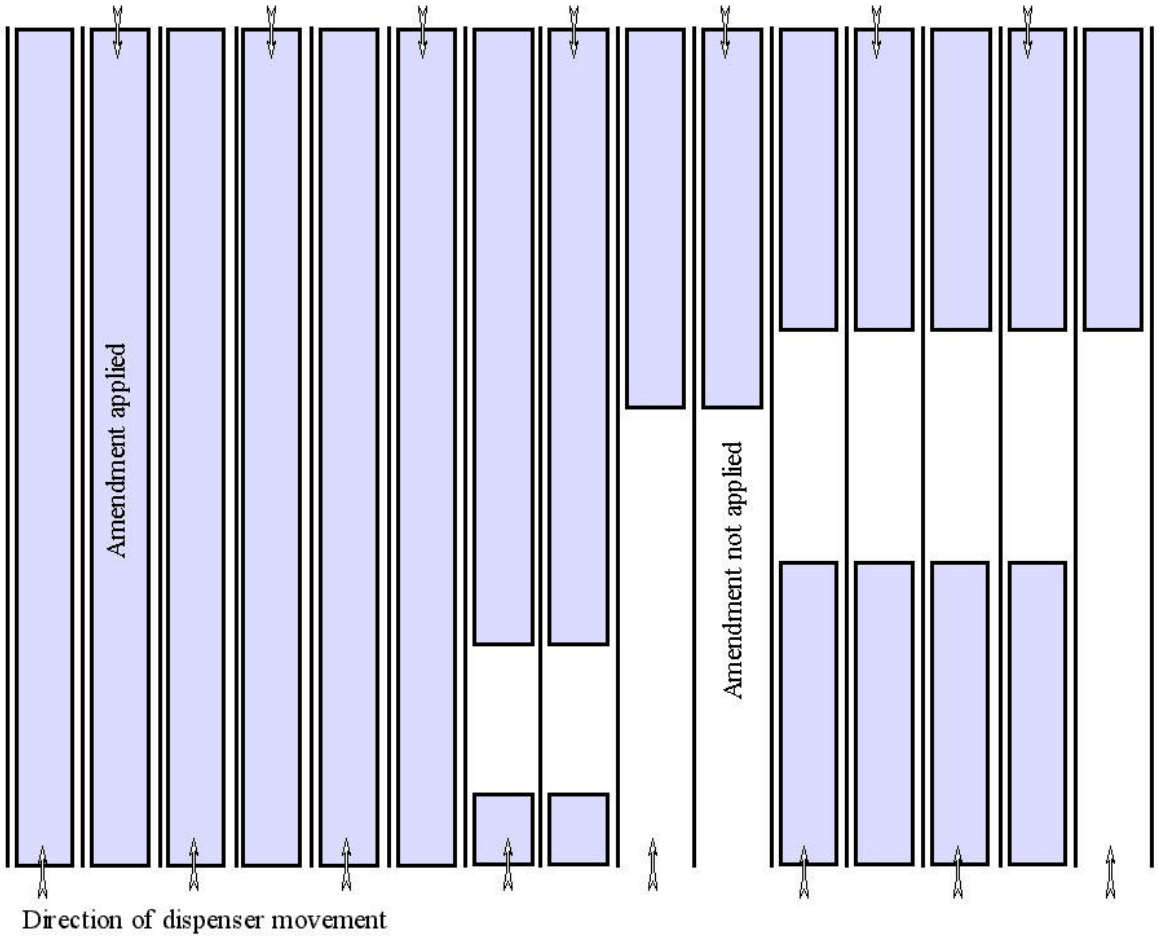

Figure 6. Scheme of amendment application on the CF2 site.

The columns describe the sequence of amendment applied/amendment not applied portions of lands a function of distance. The total treated distance was 15 rows x110 m/row = 1650 m, within which the distance not treated due to low metal concentration in soil was 350 m. In Figure 6, the negative values indicate the untreated distances in each of the 110 m rows. This means that 21% of the total distance was not treated. According to routine procedure, samples of soil and plant material after treatment were taken for analysis of metal content.

Figure 7. New generation dispenser tested at CF2.

Laboratory and parking lot testing of the equipment was completely successful. During the initial (Crop 1) full field tests, opportunities for improvement of the system were identified and were implemented prior to Crop 2. Final tests of the computer-driven dispenser were completed on Crop 2, which was grown at the CF2 site and was treated on 21 September.

The same digital map of soil contamination was used for the second crop. As a target value for the decision to apply/not apply amendments, the concentration of 300 mg Pb/kg in soil was used again. The distance each row receiving amendments were the same as used previously (see Table 1).

The total treated distance during crop 2 was 15 rows x110 m/row = 1650 m within which the distance not treated due low metal concentration in soil was 350 m. This means that 21% of the total distance was not treated. The schematic working diagram for amendment application on the site is presented previously in Figure 6.

During the actual field application of the dispenser control system, tests for accuracy were performed periodically. The results demonstrated that over a distance of 110 m the value estimated by the computer was within ±1 m of the actual value. Therefore, the error on the distance evaluation is about 2%.

According to routine procedures, samples of soil and plant material were taken after treatment and will be analyzed for metal content.

Conclusion

The cost analysis performed last year estimated a process cost of 11 USD per square meter. 71% of this estimate consisted of amendment costs. Applying amendments in relation to soil lead concentration results in savings of approximately 1.64 USD per square meter (16400.00 USD/ha).

It was estimated that during the course of the project, the major cost factor (i.e., soil amendments) was reduced by as much as 50%, resulting in overall savings of 35%. FY01 plans call for a full-scale deployment of the current generation dispenser system. Cost and performance data will be collected to document the operational parameters of this device.

WSRC-TR-2001-00106

Project Deliverable

Phytoremediation Project

FY00 Final Report – Task 5

Evaluation of Novel Mercury Remediation Technology

Submitted to:
U.S. Department of Energy
National Energy Technology Laboratory
Morgantown, West Virginia

Submitted by:
Institute for Ecology of Industrial Areas
Katowice, Poland

Institute for InternationalCooperative Environmental Research
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November 2000

TABLE OF CONTENTS

Introduction

Anthropogenic mercury emissions into the atmosphere and atmospheric deposition have increased significantly since pre-industrial times, especially in the northern hemisphere (Staines 1991, Slemr and Langner 1992[1]). In the last decade, atmospheric mercury emission in the world was estimated to be between 2000 to 4000 Mg/year. In the 1990's in Poland the estimated volume of mercury emission was approximately 35 Mg/year. The principal sources of atmospheric mercury emission in the United States are fossil fuel and medical waste incineration, which collectively account for >80% of all anthropogenic sources. In Poland the main sources of atmospheric emissions are:

- burning of fossil fuels, mainly coal;
- cement production;
- industrial production processes, in particular the mercury cell chlor-alkali processes for production of Cl and caustic soda;
- smelting of ferrous and non-ferrous metals, in particular Cu and Zn smelting;
- consumption-related discharges (light industry, dental and other).

DOE sites in the United States and industrial sites in Poland have similar forms of mercury contamination and similar affected media. In fact, the Oak Ridge mercury problem is one of the more serious situations in the U.S., which may benefit from this study. Therefore, an extensive evaluation of available remediation was performed.

The purpose of this project was to identify and evaluate promising technologies for the remediation of mercury-contaminated soil. The technology addressed in this report binds mercury and its compounds in soil, using combined chemical and biological methods, and then maintains soil conditions that inhibit the mobility of mercury. During the first year of the project an intensive literature review was conducted and soil contamination was characterized at polluted sites.

Mercury and its compounds in soil

(for details see Appendix 1)

Natural background levels

Natural background levels of mercury in soils and sediments vary between 20 and 500 µg/kg, but are generally below 100 µg/kg (Lindsey 1979, Adriano 1987). These values tend to increase with organic carbon content. In rock types containing cinnaber or other mercury-rich minerals, much

higher concentrations can occur. Natural background levels in groundwater are generally below 0.05 µg/L. A number of investigators found a highly significant correlation between mercury and organic matter content in the surface soils (Kolka at el. 1999).

Concentration levels in polluted soils

Few published data exist on mercury content in contaminated soils, although mercury contamination in soil is a problem found at many production (by active and inactive) sites. Sources of contamination include the following industrial processes:

- chlor-alkali industries, where elemental mercury was used as an amalgam- cathode;
- acetaldehyde industries, where $HgSO_4$ was used as a catalyst;
- vinyl chloride production facilities, where activated carbon with $HgCl_2$ was used as a catalyst;
- gold mining.

Critical concentrations for contaminants in soils

When determining the critical limits for contaminated soils, the following factors should be taken into account:

- both ecological- and human toxicological considerations;
- natural soil conditions and existing concentrations of contaminants;
- special parameters such as the type, composition, permeability, chemical and physical properties of soil, since they are related to the content of organic matter and clay in the soil;
- the limits should be mutually consistent for soil/sediment and for groundwater.

The literature studies conducted for this project identified standards for mercury content in water and soil from various countries.

In Poland the permissible concentrations of mercury in soil depending on the character of the area are as follows:

- 0.3 - for legally protected areas , areas of underground therapeutic waters, catchment areas for underground water reservoirs and protection zones of springs and intakes of underground waters (Zone A);

[1] See Apendix 1.

- 3.0 - for areas used for agricultural purposes, forests, housing recreation and other places of public use (Zone B); and
- 30.0 - for industrial plants, liquid and solid fuel storage areas, communication routes, waste disposal sites, and airports (Zone C).

Review Of Soil Treatment Technologies

Various methods have been tested for the removal of mercury from contaminated environmental media. These methods include liquid extraction, thermal treatment, electrolytic methods, mercury flotation, mercury immobilization, precipitation of mercury ions and flotation of precipitants. For the purpose of this report, applicability of the technologies has been evaluated using the following criteria:

- effectiveness of mercury removal;
- mercury content in the soil;
- potential mercury migration in the environment;
- cost effectiveness; and
- methods for the management of waste generated by the technology applied.

Commercially-available technologies for removing mercury from soil include:

- chemical treatment/stabilization,
- thermal desorption/destruction,
- chemical extraction,
- soil washing/soil flushing,
- electrochemically remediation, and
- bioremediation and phytoremediation.

Summary of the literature review

This section summarizes an extensive literature review that is presented in Appendix 1. This literature review focuses on remediation technologies for soil contaminated with mercury. We found that current available technologies have a number of significant drawbacks including:

- high capital costs;

- concentration limitations, these technologies are effective only at extremely high mercury concentrations;
- limited experience with soil clean-up, most of these technologies have been applied for waste, water and sludge remediation rather than soil; and
- complexity, most of the technologies include multiple phases which require multi-phases requiring preparation and pre-treatment of the target media.

It can be concluded that none of the technologies studied and presented in this report have been identified to be effectively applied to cleanup soils contaminated with mercury. According to the latest literature data, the current approaches to soil remediaton of heavy metals focus on the use of plants and microorganisms as these methods are cost effective and environmentally friendly. Mercury remediation from soil is an area of current interest and in which there is no established baseline technology.

The data from the up-to-date laboratory experiments is promising and seems to provide further support to the applicability of phytoremediation for soil clean-up. However, attention should be paid to the problem of safe handling of the contaminated crops.

Based on the findings of our literature review, it was decided that a field site should be selected on which to evaluate potential remedial technologies. Such a site was located and arrangements were made with the site owners to conduct preliminary site characterization activities for mercury contamination.

Site Identification

The site in question is a chemical facility known as AZOTY, where mercury and its compounds are used in multiple manufacturing processes. The AZOTY chemical works in Tarnow, Poland, has been operating for over 70 years, and has become a well-recognized chemical enterprise in both domestic and international markets. The facility produces approximately 100 products. Mercury is used in the production polyvinyl chloride and chlor-alakli prodution.

Site Characterization

There are three sources of mercury emission to the atmosphere from the AZOTY Site: chlorine production using electrolysis, recovery of mercury from waste materials and coal combustion in the facility's power and heating plant. Annual mercury emission volumes from chlorine production are presented in Table 1.

Table 1. Mercury emission volumes [kg/year]

	1991	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**
Mercury	1754	218	198	202	172	163	203	91	55

Data analysis from the facility's long term environmental monitoring programs reveals that the area in the vicinity of the AZOTY facility has not been contaminated with mercury from large-scale airborne deposition. Data for annual emission, mercury deposition and surface water concentrations (Dunajec and Biala Rivers) indicate that small scale direct mercury-contamination at the AZOTY Facility site has not spread to surface water or groundwater. Thus, the contaminated area is enclosed within the facility boundaries.

Mercury contents in soil

On the basis of the investigation of mercury content in soil at the AZOTY Plant carried out in the period of 1994-1995 and again in 2000, four mercury-contaminated sites were identified:

- Electrolysis cells chlorine production,(approx. 2500 m^2);
- Mercury regeneration facility (for recovering mercury from process waste) (approx. 300 m^2);
- PVC and vinyl chloride production installation (approx. 1000 m^2); and
- incineration site where discarded chemical equipment was disposed and anticorrosive coatings were incinerated in an uncontrolled way (approx. 15 000 m^2).

Soil was sampled for analysis from the following depths:

- 0-20 cm (top layer),
- 40-60 cm,
- 90-110 cm, and

ÿ 140-160 cm.

The results of the total mercury concentrations (median) in individual layers and four sites of mercury contamination are presented in Table 2.

Table 2. Total mercury concentration [mg/kg of dry matter] in soil by depth .

Depth (cm)	Electrolysis Cells		Mercury regeneration facility		PVC - production facility		Incineration site	
	Range (n=20)	Median	Range (n=4)	Median	Range (n=7)	Median	Range (n=28)	Median
0 - 20	4.09 - 165.5	40.0	410.1 - 821.0	557.0	21.28 - 830.5	261.6	5.97 - 3663.0	161.5
40 - 60	2.04 - 49.18	9.83	10.57 - 13.36	12.23	0.67 - 44.53	9.13	37.86 - 133.2	37.86
90 - 110	0.40 - 13.15	4.95	1.36 - 47.75	18.55	0.28 - 7.26	2.23	1.94 - 45.57	12.18
140 - 160	0.48 - 7.36	2.60	1.47 - 6.55	3.28	0.25 -1.32	0.93	0.55 - 13.98	4.90

The greatest mercury concentrations are found in surface soils and decrease rapidly with depth. However, the vertical distribution of mercury concentrations in soil and mercury concentrations in the piezometer samples indicates the downward migration of mercury. This movement of mercury is cause for concern and warrants efforts to remediate this problem. In view of the technical limitations in mercury removal technologies, it was decided to focus our efforts on the immobilization of mercury and its compounds. At present, our research is focused on the selection of chemical substances and plants that would effectively bind/stabilize mercury and its compounds in soil.

Mercury speciation

The selection of stabilizing agents (chemical compounds, plant material, microorganisms) or mercury-binding compounds in the soil requires knowledge of mercury speciation in addition to total mercury concentration.

The following sequential speciation procedure for the determination of mercury species was applied in addition to standard analysis of total mercury:

ÿ water soluble-extraction with deionized, distilled water;

- exchangeable-extraction with1M CH_3COONH_4;
- fulvic-bound and humic-boundextraction with 1M KOH and subsequent acidification to pH 1÷2 with HNO_3;
- organic/sulfide bound extraction with 0.1M HNO_3 and H_2O_2; and
- residual-digestion with aqua regia.

Mercury concentration was determined by the cold vapor AAS method.

The results of sequential speciation analysis of mercury species in soil are presented in Table 3.

Table 3. Data from the sequential speciation analysis of mercury in soil [mg/kg dry matter] (n=10)

Mercury forms	Range	Mean	SD
Water soluble	0.363 - 0.567	0.437	0.082
Exchangeable	2.405 - 3.795	3.092	0.61
Fulvic and humic bound	27.5 - 40.66	33.44	4.19
Organic/sulfide bound	95.88 - 119.2	106.1	9.58
Residual	98.76 - 128.6	110.4	9.79

The mean total mercury content in the analyzed soil sample was 261.5 mg/kg ± 9.21 mg/kg (dry matter) - range 250.3 - 273.7 mg/kg (dry matter).

Binding compounds and plant species were selected carefully with consideration of physical and chemical properties of the soil as well as mercury concentration. The primary goal was to maintain soil pH within a range that immobilizes mercury.

Mercury Resistant Plant Species Screening of Soil Microbial/Fungi Activity and Stabilization Using Plants and Microbes

This section summarizes a technical report that is presented in Appendix 2. Living plants have the ability to accumulate heavy metals, in particular those metals that are essential for growth and development, from soil/water solution. Certain plants also have the ability to accumulate heavy metals, which have no known biological function (e.g., Pb, Cd, Hg). However, excessive accumulation of these metals can be toxic to most plants. Heavy metal ions, when present at elevated levels in the environment, can be adsorbed by roots and translocated to different plant parts, however, this typically leads to impaired metabolism and reduced growth.

The roots of plants interact with a large number of different microorganisms. Microbial populations are invariably higher in the rhizosphere than in root-free soil. Populations of bacteria in field soils may exceed 100 million g^{-1} as estimated by soil dilution and plate counts. From such counts the rhizosphere/non-rhizosphere microbial population ratio (R/S) values are most frequently in the range of 2–20. The ratio of fungal to bacterial biomass in arable soil was found to be 5–10,

and the ratio percent root surface covered by fungi/percent covered by bacteria has been estimated to range from 0.28–14.0.

Pseudomonas spp. Are prevalent in the rhizosphere and both *P. fluorescens* and *P. putida* are known to enhance plant growth. Soil actinomycetes, predominantly *Streptomycess* spp., are usually second to bacteria in populations, with common R/S ratios of 5 to 10. Actinomycetes are best known for antibiotic production and the inhibition of plant pathogens and other soil microorganisms.

In general, populations of fungi are lower than those of bacteria and actinomycetes. The R/S ratios derived from plate counts may range from 3:1 to > 100:1 but most frequently are 10:1 to 20:1 for crop plants.

Some bacteria can convert Hg^{2+} (a highly reactive and toxic water-soluble ionic form of mercury) into Hg^{o}. The elemental form of mercury is much less toxic than the mercurous (Hg^{2+}) form, and the physical properties of Hg^{o} greatly decrease its microbial availability. Hg^{o} is virtually insoluble in water and has a high vapor pressure, which leads to rapid volatilization. This mechanism is induced at the picomolar mercury concentrations found in natural systems (water/soil).

The generally accepted mechanism of bacterial mercuric ion resistance generally accepted involves intracellular reduction of Hg^{2+} to Hg^{o} by mercuric reductase, with a subsequent volatilization. Mercuric reductases have been isolated from a number of microorganisms, including *Escherichia coli, Thiobacillus ferrooxidans*, *Streptomyces*, *Streptococcus*, and *Caulobacter*. Bacteria have an important role in mercury cycling, with acidification leading to increased bacterial methylation of mercury and subsequent bioaccumulation in higher organisms.

The goal of the task was to screen the microbial communities associated with different plant species for their reaction to mercury-contaminated soils. The following were investigated during this task: the types of microorganisms; their reaction on mercury-contaminated soil; of the number of microbes in rhizosphere vs. root-free soil (R/S) for different plant species. The plant experiments were performed in growth chambers monitored for light and temperature.

Table 4. Soil properties are presented

Investigated Component	Unit	Mean = SD
pH_{H2O}		7.70 = 0.00
pH_{KCl}		6.92 = 0.02
Electrical Conductivity	µS/cm	201.5 = 1.5
Cation Exchange Capacity	mol/kg	15.27 = 1.36
Organic Carbon	%	1.77 = 0.14
Organic Matter	%	3.05 = 0.23
Total:		
N	%	0.105 = 0.00
P_2O_5	mg $100g^{-1}$	0.137 = 0.01
K_2O	mg $100g^{-1}$	12.2 = 0.11

Water regime was maintained at 60% of total water capacity. Because of the high content of soil phosphorus and potassium, only nitrogen fertilizer was applied. Species utilized in the experiment are presented below. Test plants were planted on mercury contaminated soil in four replications.

Fertilization	
mercury-contaminated soil planted with plants A, B, C, D, E	**mercury–contaminated soil blank**
plant A: *Helianthus tuberosus* plant B: *Armoracia lapathifolia* plant C: *Poa pratensis* plant D: *Festuca rubra* plant E: *Salix viminalis*	

Growth, yield, health and vitality of investigated plant species were reported, as well as mercury distribution in different plant parts.

Time of root rhizosphere development was established as one month. The soil was sampled inside and outside of the rhizosphere from each pot. Samples were collected twice-after one month (7 of July), and in the end of experiment. Standard garden soil was used as a control medium for microbiological investigation. Samples were microbiologically examined for:

- total number of soil bacteria [10% tryptic soy agar (TSA; Difco)];
- number of Pseudomonas on the selective medium (Grant and Holt, 1977);
- number of soil fungi on Czapek-Dox medium with Rose Bengal (Alef, 1995);
- number of protein decomposing bacteria (by the MPN methods);
- number of sulfur amino-acid decomposing bacteria;
- number of ammonificators; and
- number of nitrificators,

Results were expressed as colony forming units per gram of dry soil (CFU g^{-1}).

All soil samples are analyzed for soil pH (H_2O and KCl) using the **ISO 10390** method.
Soil samples from each container were divided into:

- root-free soil - 10 g of root-free soil was transferred to 250 mL Erlenmeyer flasks containing 90 mL 0.85% NaCl. Flasks were shaken on a rotary shaker for 10 min at 120 rev min^{-1}. The soil suspension was used for the determination of the root-free soil population; and
- rhizosphere soil - plant shoots were removed from the soil, roots with adhering soil were cut into small pieces. Samples of 5 g roots were transferred to 250 mL Erlenmeyer flasks containing 45 mL 0.85% NaCl. Flasks were shaken on a rotary shaker for 10 min at 120 rev min^{-1}. Soil suspension was used for the determination of the rhizosphere population.

Results are shown in Table 3 of Appendix 2 Roots of plants (*Poa pratensis* and *Festuca rubra*) interact with a large number of different microorganisms (e.g. *Pseudomonas, Streptomyces***,** soil fungi, protein-decomposing bacteria). Generally microbial populations were higher in the rhizosphere than in the root-free soil. Results of experimental investigation can be summarized as follows:

- Plant growth and development on mercury-contaminated soil was comparable to the reference garden soil.
- Investigated plants accumulate mercury compounds mainly in their roots.

- The highest amounts of mercury were found in willow (*Salix viminalis*) roots. However, willow's root systems are located mainly in the upper layer of mercury contaminated soil, which is a disadvantage in terms of soil stabilization processes.
- Both grasses that were studied (meadow–grass and fescue) accumulated smaller amounts of mercury when compared to the willow, but created a good soil penetration and stabilization system.
- The highest numbers of *Pseudomonas* bacteria were found in the rhizosphere of both grasses and willows. The number of *Pseudomonas* was higher in the rhizosphere when compared to the root-free zone. Most probably *Pseudomonas* development was supported by special root excretion.
- Mercury-contaminated soil microflora investigations have shown the variability in physiological groups and taxa of bacteria. The soil, although contaminated with mercury, promotes plant growth.
- Plants are able to develop a rich rhizosphere zone in mercury contaminated soil, however, the plant's roots appear to exclude mercury. Both phenomena are essential for soil stabilization processes using plants.

Mercury Stabilization in Soil Using Chemicals

(for more details see appendix 1)

The goal of this subtask was to select appropriate chemical substances to stabilize/bind mercury and its compounds. Such stabilization is needed in order to prevent the migration of mercury to other environmental receptors, especially groundwater.

In order to achieve this objective lab-scale experiments were carried out to identify a chemical substance that most effectively binds or stabilizes mercury and its compounds in soil. Changes in mercury content in the soil as a function of time also were determined. The following chemical substances were and are currently being tested:

- fine sulfur,
- granular sulfur,
- zeolites, and
- mixtures of dolomite and zeolite (10:1) with an addition of brown coal.

The experimental plan was as follows:

1) mercury contaminated soil-blank (3 replicates);
2) mercury contaminated soil + fine sulfur in the amount 0.5%, 1% and 5%) (3 replicates);
3) mercury contaminated soil + granular sulfur in the amount 1%, 5% and 10% (3 replicates);
4) mercury contaminated soil + zeolite in the amount 1%, 5% and 10% (3replicates); and
5) mercury contaminated soil + mixture dolomite and zeolite with brown coal in the amount 1%, 5% and 10% (3 replicates).

- Soil sampling occurred every two weeks (8-10 sampling campaigns),
- Mercury content in soil was determined for the following forms:
  - water soluble,
  - exchangeable,
  - bound with fulvic and humic acid.

Twenty weeks after the addition of various amendments to the mercury-contaminated soil, the fraction of easily soluble mercury forms in water was reduced. It was found that the type and quantity of added chemical substances had no significant effect on stabilization or binding of water soluble mercury compounds.

The same phenomena were observed in the case of compounds bound with exchangeable forms. Similarly, the type and quantity of the added chemical substances had no significant effect on the stabilization or binding of exchangeable mercury compounds.

In the case of mercury compounds bound with fulvic and humic acid, no statistically significant differences were observed after the addition of various stabilization/binding substances in similar time .

Results indicate that the chemical substances added to soil stabilize water soluble and exchangeable mercury compounds in soil after 6 weeks.

Summary

The goal of this task was to develop a technology to bind and immobilize mercury and its compounds in soil, using inexpensive chemical substances and then to establish soil conditions that maintain mercury in immobilized forms. In addition microflora association with plant roots inhibits chemical reduction mercury.
The current best available control technologies (BACT), combine chemical/biological components. New bioremediation technologies for mercury stabilization in soils, combine the activity of chemical compounds, microorganisms and plants, to effectively confine the contaminant.

In order to achieve mercury stabilization in soil, lab-scale experiments were conducted. Common inexpensive chemical substances (i.e. sulfur, zeolite, biodecol), which are known for their ability to bind metals, were investigated. Changes in mercury content in the soil as a function of time also were determined.

In case of addition of 0.5% granular sulfur to soil, 78% of water soluble and exchangeable mercury fraction was bound while in case of zeolite addition to soil 49% of these fractions was bound.

The addition of high concentration granular sulfur had no significant effect on the stabilization or binding of water soluble and exchangeable forms of mercury compounds. We suspect that a decrease in pH after sulfur addition to the mercury-contaminated soil resulted in precipitation of insoluble mercury compounds.

Plants studied to date for use in mercury remediation accumulate mercury compounds mainly in their roots. The highest amounts of mercury were found in the roots of willow (*Salix virminalis*) Both investigated grasses (meadow-grass and fescue) have accumulate less mercury when compared to the willow, but created a good soil stabilization system though their root mats.

The microflora associated with established root mats will play an important role in maintaining stable soil conditions that inhibit mercury transport. The highest numbers of *Pseudomonas* bacteria responsible for mercury detoxification, i.e. were found in the rhizosphere of both grasses and willow. The soil, although contaminated with mercury, supported plant growth and plants developed a rich rhizosphere zone in mercury contaminated soil. Both phenomena are essential for

soil stabilization processes using plants. Based on presented results, grasses seem to be appropriate species for stabilizing mercury-contaminated soils.

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Project Deliverable

Phytoremediation Project

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Evaluation of Novel Mercury Remediation Technology

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Introduction

This report presents the results of a U.S. Department of Energy sponsored project that was carried out by Florida State University and the Institute for Ecology of Industrial Areas, Katowice, Poland. The purpose of the project, entitled „Evaluation of Novel Mercury Remediation Technology" was to identify and evaluate promising technologies for the remediation of mercury-contaminated soil. The technology addressed in this manuscript binds mercury and its compounds in soil, using an inexpensive substance, and then maintains soil conditions that inhibit the movement of mercury. During the first year of the project an intensive literature review was conducted and soil contamination was characterized at polluted sites.

The site in question is a chemical facility known as AZOTY, where mercury and its compounds are used in multiple manufacturing processes.

Anthropogenic mercury emissions into the atmosphere and atmospheric deposition have increased significantly since pre-industrial times, especially in the northern hemisphere [1, 2]. In the last decade, mercury emissions to the atmosphere in the world was assessed on the level of 2,000 to 4,000 Mg/year [2]. In the 90's in Poland the assessed volume of mercury emission into the atmosphere was approximately 35 Mg/year [3]. The principal sources of atmospheric mercury emission in the United States are fossil fuel and medical waste incineration, which collectively account for > 80% of all anthropogenic sources [4]. In Poland the main sources of atmospheric emissions are:

- burning of fossil fuels, primarily coal,
- cement production,
- industrial production processes, in particular the mercury cell chlor-alkali processes for production of Cl_2 and caustic soda,
- smelting of ferrous and non-ferrous metals, in particular Cu and Zn smelting, and
- consumption-related discharges.

Anthropogenic contributions to the mercury burden of ecosystems can result in bio-concentration levels toxic for biological systems. For instance, the increase of the mercury load of soil, lakes and rivers, caused by direct atmospheric deposition via airborne mercury deposited on soil of the catchments [5, 6, 7]. Behavior of mercury and its compounds in the soil depends on two contradictory phenomena:

- sorption through mineral and organic sorptive complex, precipitation of insoluble compounds, and biogenic cumulation leads to accumulation of mercury and its compounds in the surface layers of soil;
- desorption, and solubility and mineralization of organic compounds increase the mobility of mercury and its compounds in soil.

Mercury contamination of soil and water is a global environmental problem, especially in the developing countries where mercury is being used increasingly. Industries of western countries are trying to reduce or eliminate the use of technologies involving mercury and its compounds due to regulatory concerns regarding mercury toxicity. Nevertheless, at numerous post-industrial sites, large amounts of mercury are contained in soils, posing significant risk to humans and the environment.

There are DOE sites that are contaminated with mercury in forms and affected media that similar to the industrial sites which are contaminated in Poland. In fact, the Oak Ridge problem with mercury may be one of more serious situations in the U.S. Therefore, an extensive evaluation of available remediation was performed.

Mercury is not an easy soil pollutant to deal with, because under natural conditions the release of Hg^o and possibly other volatile mercury compounds from soil is probably significant in the cycling of mercury between various compartments of the environment.

The purpose of this project was to identify and evaluate promising technologies for the remediation of mercury-contaminated soil. The technology addressed in this report binds mercury and its compounds in soil, using an inexpensive substance, and then maintains soil conditions that inhibit the movement of mercury.

Mercury and its compounds in soil

Natural background levels

Natural background levels of mercury in soils and sediments vary between 20 and 500 µg/kg, but are generally below 100 µg/kg [8]. These values tend to increase with the C_{org} content [9,10]. In

rock types that contain cinnaber or other Hg-rich minerals, much higher concentrations can occur. Natural background levels in groundwater are generally below 0.05 µg/l [9, 10]. Table 1 identifies typical mercury levels in uncontaminated soils [11, 12, 13].

Table 1. Mercury contents of surface soils on the world (µg/kg dry matter) [11, 12, 13]

Type of soil	Rangeµg/kg	Averageµg/kg
Psamments	8-700	50
Loam	10-1000	100
Organogenic	40-1110	260
Rendolls	10-500	50
Chernozems	20-530	120
Forest	20-580	140
Desert	8-320	70
Volcanic	1.4-180	30
Others	4-990	110

A number of investigators found a highly significant correlation between mercury and organic matter content in the surface soils [9, 10, 14, 16, 17,18].

The most significant chemical species of mercury participating in the geochemical cycle of this element may be classified as follows [1,15]:

- volatile compounds Hg^0, $(CH_3)_2Hg$,
- reactive species Hg^{2+}, HgX_2, HgX_3^- and HgX_4^{2-} with X =OH^-, Cl^- and Br^-, Hg^{2+} complexes with organic acids, and
- non-reactive species such as methyl mercury and other organomercuric compounds, HgS, Hg^{2+} bound to S atoms in humic matter.

Figure 1. Mercury compounds in soil and the chemical reaction, R-radical alkyl (CH_3)[11]

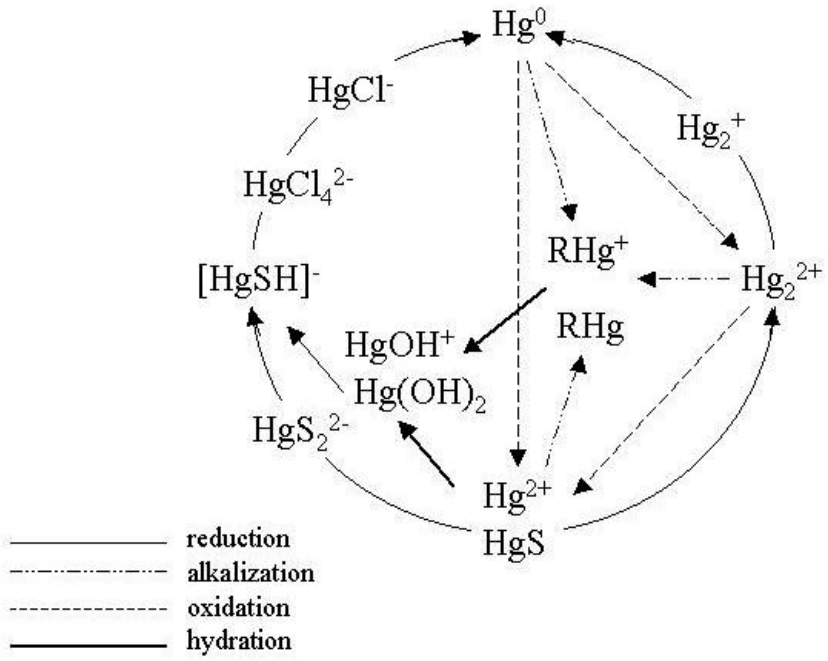


Fig. 1. Mercury compounds in soil [11]

Concentration levels in polluted soils

Most data on polluted soils concern river and lake sediments. In the Netherlands, mercury contents are recalculated to a 50% < 16 µg fraction because most of the mercury is bound to this fraction. In contaminated river sediments, concentrations up to 6 mg/kg (Rhine) were noted in the Netherlands, and in Germany up to 20-30 mg/kg (Elbe and Rhine) [9].

There are few published data on mercury contents in contaminated soils. The problem of mercury contamination in soil is faced at many inactive and active production sites. Sources of contamination include the following technical processes [19, 20, 21, 22, 23]:

- chlor-alkali industries, where elemental mercury was used as amalgam-cathode,
- acetaldehyde industries, where $HgSO_4$ was used as a catalyst,
- vinyl chloride productions, where activated carbon with $HgCl_2$ was used as a catalyst,
- gold mining.

Soil contents on the order of few to several thousands mg/kg Hg were founded in the vicinity of manufacturing processes where mercury and its compounds are or were used [19, 20, 21, 22, 23]. Around an inactive building of a chlor-alkali production complex in the former German Democratic

Republic, values on the order of 27-46,000 mg/kg have been observed in soils [22]. Thermal analyses demonstrated, that samples with high amounts of total mercury (up 465 ppm) do not show the presence of Hg^0, but likely bound only to humic substances [22]. Results obtained from the sequential leaching procedure for soil samples from the site of former chlor-alkali are the following [24]:

- water soluble - 0.13-0.15%,
- exchangeable - 0.72-0.97%,
- humic/fulvic - 8.6-11.1%,
- organic/sulfide - 47.3-49.4%,
- residual - 38.4-43.5%.

Having established that an area of soil is contaminated, it is necessary to make a decision about the action that needs to be taken in order to avoid unnecessary risk of health or damage to structures. For this purpose, various sets of critical concentrations are in use around the world.

Critical concentrations for contaminants in soils

When determining the critical limits for contaminated soils, the following aspects should be taken into account [25, 26, 27, 28]:

- both human toxicological and eco-toxicological considerations,
- natural soil conditions, existing concentrations of contaminants
- special parameters such as type of the soil, its composition, permeability, chemical and physical properties since they are related to the content of organic matter and clay in the soil,
- the limits should be mutually consistent for soil/sediment and for groundwater.

The literature studies also revealed standards for mercury content in water and soil used in various countries. These standards for mercury content in water and soil are listed in Table 2 [25, 26, 27, 28, 29].

Table 2. Standards for mercury content in water and soil [25, 26, 27, 28, 29].

Country	Surface water [µg/L]	Groundwater [µg/L]	Soil [mg/kg dry material]
United States	0.012-2.4		
Canada	0.1		2.0-30.0[1]
The Nertherlands	0,23	0.05-0.3	0.3-10.0[1]
Germany	0.8		1.0-10.0[1]
United Kingdom			1.0-20.0[1]
Germany	0.8		
Denmark	1.0		
Poland	1.0	0.05-2.0[*]	1.0-30.0[1*]
Czech Republic	1.0	2.0-5.0[1]	0.6-20.0[1]
Slovenia	1.0		0.8-10.0[1]

[1] intervention values (critical values)

[*] Guidelines of the State Environmental Protection Inspection

In Poland there are neither standards nor legal regulations or recommendations which determine permissible concentrations of chemical substances in soil. In order to make a direct assessment of the level of groundwater and soil contamination in the environment, the Polish State Environmental Protection Inspection has developed a list of chemical substances together with their permissible concentrations [29].

The permissible contents of mercury in soil in [mg/kg dry matter], depending on the character of the area are as follows:

- 0.3 - for areas legally protected, areas of underground therapeutic waters, catchment areas of the used underground water reservoirs and protection zones of springs and intakes of underground waters (Zone A),
- 3.0 - for the areas used for agricultural purposes forests, housing recreation and other places of public use (Zone B), and
- 30.0 - for industrial plants, fluid and solid fuels storage areas, communication routes, waste disposal sites, airports (Zone C).

Once an area of land has been identified as being contaminated, it becomes necessary to decide what action should be taken with regard to the restrictions on its use and/or requirements for the amelioration or „clean-up" of the soil. There are several options available for remediation of contaminated sites. The decision will be based upon on the nature of the contaminants, the type of soil, the characteristics of the site, its intended use, the relative costs of the appropriate options, and the regulations which apply in the country where the site is located. The remediation options may vary from the minimum of reducing the bioavailability of the contaminants, to the maximum of either complete clean up of the soil, or its removal from the site.

Review of soil treatment technologies

Various decontamination methods such as liquid extraction, thermal treatment, electrolytic methods, mercury flotation, mercury immobilization, precipitation of mercury ions and flotation of precipitation are being studied. The available technologies have been evaluated from the point of view of their effective application as the primary or supporting process to mercury-contaminated soil remediation by phytoremediation as well as a technology competitive to phytoremediation.
Applicability of the technologies has been evaluated using the following criteria:

- effectiveness of mercury removal,
- mercury content in soil,
- potential mercury migration to other environmental components,
- cost effectiveness, and
- methods for the utilization of waste generated by the technology applied.

There is a number of commercially available mercury soil cleaning technologies, including:

- chemical treatment/stabilization,
- thermal desorption/destruction,
- chemical extraction,
- soil washing/soil flushing,
- electrochemically remediation,
- bioremediation and phytoremediation.

Chemical treatment

Chemical treatment processes [30]: convert hazardous constituents into less toxic, less objectionable environmental forms in order to meet treatment objectives. They consist of a series of techniques that can be selectively applied to destroy or modify organic or inorganic contaminants. The selection varies depending on the particular contaminants and media. Chemical treatment is rarely used alone. It is usually employed as a pre- or post-treatment process in site remediation and seldom as a stand-alone process.

Among chemical treatment processes which have been applied for site remediation (Substitution, Oxidation and Precipitation) only Precipitation may be considered for treating materials containing metals. Precipitation processes, by their nature, are limited to liquid systems - they are routinely used in treating wastewater. Their application in site remediation is less common but it appears to have potential for removing toxic material.

In treating soils it would normally be classified as a stabilization rather than a chemical treatment process. This treatment system is capable of chemically destroying certain chlorinated organics and immobilizing heavy metals. There is a fine distinction between chemical precipitation and stabilization/ solidification (S/S) operations. In S/S operations, the contaminants are incorporated into a cement-like matrix, rendering the contaminants less prone to leaching. Sludges are chemically treated by mixing a binder material to improve the physical and chemical stability of the sludge.

One potential limitation lies in the collection and handling of precipitates.
Stabilization and solidification are closely related in that they both use chemical, physical and thermal processes to detoxify hazardous contaminants. However, they are distinct methods defined as follows [31]:

- Solidification - processes that encapsulate the contaminated soil in a monolithic solid of high-structural integrity and immobilized the harmful constituents. Solidification does not necessarily involve a chemical interaction between the waste in the monolith. Contaminant migration is restricted by vastly decreasing the surface area exposed to leaching and/or by isolating the waste within an impervious capsule.

ÿ Stabilization - processes that convert the contaminants into a less soluble, mobile or toxic form in which the physical nature of the waste is not necessarily altered. These processes reduce the risk posed by a contaminated soil.

Examples of technologies:

Chemical Treatment and Immobilization [32]: This solidification technology may be applied for mixed hazardous wastes, cement or fly ash, water and one of the 18 patented reagents commonly known as "Chloranan". In the case of chlorinated organics, the process uses metal-scavenging techniques to remove chlorine atoms and replace them with hydrogen atoms. Metals are fixed at their lowest solubility point. Soils, sludges and sediments can be treated *in situ* or may be treated *ex situ*. Sediments can also be treated underwater. Blending is accomplished in batches, with volumetric throughput rates of 120 tons/hr.

The treatment process begins by adding Chloranan and water to the blending unit, followed by untreated waste and mixing for 2 minutes. The cement is added and mixed for a similar amount of time. After 12 hours, the treated material hardens into a concrete-like mass that exhibits unconfined compressive strengths (USC) in the 1000 to 3000 pounds per square inch (psi) range with a permeability of approximately 10^{-9} centimeters. Results may varyasthis material is capable of withstanding several hundred cycles of freeze and thaw weathering.

This technology has been refined since the 1987 SITE demonstration and is now capable of destroying certain chlorinated organics and also immobilizing other wastes, including high levels of metals.

Costs are estimated at 40 to 60 US dollars/ton for processing heavy metal waste (75 –100 US dollars/ton for waste with organic contents).

Modified Sulfur Cement [31]: Modified sulfur cement is a commercially-available thermoplastic material. It is easily melted (127 ^{o} to 149oC) and then mixed with the waste components to form a homogenous molten slurry. The molten slurry is discharged to suitable containers for cooling, storage and disposal. A variety of common mixing devices, such as paddle mixers and pug mills, can be used.

Modified sulfur cement was developed by the US Bureau of Mines in 1972 as a means of utilizing waste sulfur from gas and petroleum distillation processes.

Previous attempts to use elemental sulfur as a construction material in the chemical industry failed because of internal stresses set up by changes in crystalline structure during cooling. By reacting elemental sulfur with hydrocarbon polymers, the Bureau of Mines developed a product that successfully suppresses the solid phase transformation and thus dramatically improves stability of the material.

Licensed commercially - Bureau of Mines - Martin Resources, Inc., Odessa, Texas.

The formulation contains a total of 5% by weight modifiers consisting of equal amounts dicyclopentadiene (DCPD) and cyclopentadience (CPD) that react with the sulfur to form long chain polymers. It has a melting point of 119^{o}C and a viscosity of approximately 25 centipoise (cp) at 135^{o}C.

Compared with hydraulic cements, sulfur cement has several advantages. One such advantage is that no chemical reactions are required for solidification, eliminating the possibility that elements in the waste can interfere with setting and thereby limit the range of waste materials that can be successfully encapsulated. Full strength is attained in several hours rather than weeks-sulfur concrete compressive and tensile strengths twice those of comparable portland concretes have been achieved. Sulfur concretes are resistant to attack by most acids and salts e.g. sulfates that can severely degrade hydraulic cement-have little or no effect on the integrity of sulfur cement. The first application of modified sulfur cement to the solidification of radioactive and mixed wastes was performed at Brookhaven National Laboratory.

Soluble Phosphates [31]: Soluble phosphates and lime have been used commercially to stabilize fly ash and mixtures containing fly ash resulting from the combustion of municipal solid waste. It has been postulated that this process may also be of use in the stabilization of other wastes heavily loaded with metals, such as medical waste ash, insulation wastes, metals smelting dusts, contaminated soils and metal contaminated sludges. It is primary effective against lead and cadmium, but may be of benefit also in controlling other toxic metals. However, it needs to be intensively tested for mercury.

The process involves the addition of various forms of phosphate and alkali to control pH as well as for the formation of complex metal molecules of low solubility. The intent is to immobilize or insolubilize the metals in the solid waste over a wide pH range. Unlike most other stabilization/solidification processes, soluble phosphate processes do not convert the waste into a solid, hardened, monolithic mass. Instead, the treated waste retains its particulate nature, remains free-flowing, and increases little in volume.

Chemfix Process [33]: This solidification and stabilization process is an inorganic system in which soluble silicates and silicate-setting agents react with polyvalent metal ions and other waste components to produce a chemically and physically stable solid material. The feed waste is first blended in the reaction vessel with dry aluminum, calcium and silica based reagents that are dispersed and dissolved throughout the aqueous phase. The reagents react with polyvalent ions in the waste and form inorganic polymer chains (insoluble metal silicates) throughout the aqueous phase. These polymer chains physically entrap the organic colloids within the microstructure of the product matrix. The water-soluble silicates then react with complex ions in the presence of a silicate setting agent, producing amorphous, colloidal silicates (gels) and silicon dioxide, which act as precipitating agents. Most of the heavy metals in the waste become part of the silicate gel. Some of the heavy metals precipitate with the structure of the silicate gel.

This technology is suitable for contaminated soils, sludges and other solid wastes. The process is applicable to electronic wastes, electric arc furnace dust and municipal sewage sludge containing heavy metals such as Al, Sb, As, Ba, Be, Cd., Cr, Fe, Pb, Mn, **Hg,** Ni, Se, Ag, Tl, Zn.

Thermal methods

Thermal desorption [34]: This process is a part of the total system used in the remediation of contaminated solid media. Thermal desorption is an ex situ means for physically separating organics from soils, sediments, sludges, filter cakes and other solid media. The contaminated material is excavated and delivered to the thermal desorber.

Treatment System schematic:

Discharge material handling system

Cooling

Dust control

Stabilization

Solid Post-treatment

↑

Pretreatment	→	**Thermal Desorber**	→	**Gas Post-treatment**
Excavation		Direct-fired rotary desorber		Organic collection/destruction
Storage		Indirect-fired rotary desorber		Particulate collection
Sizing		Conveyor, and others		Acid gas removal
Crushing, dewatering, neutralization,				
Blending				
Feeding systems				

In a desorber unit, heat is transferred to the solid media. The contaminated material is heated and water and the contaminants are devolatilized. In any thermal desorption system, heat must be transferred to the solid particles to vaporize the contaminants from the particles; in turn, the vaporized contaminants must be transferred from the particles to the gas phase. Researches have identified several important variables that need to be considered in the equilibrium between contaminants and soil particles. They have demonstrated that contaminant removal is highly dependent on the following parameters:

- temperature - modest increases in temperature greatly decrease residual concentrations,
- soil matrix - coarse particles such as sands will desorb contaminants more easily than fine grained clay and silts,
- contaminants - some contaminants will bind strongly to soil while others will not, and
- moisture content - increased moisture reduces the capacity of the contaminant to adsorb on soil with high mineral contents (silts and clays).

While the initial 90% of a contaminant might be easily removed, the final 10% will take much longer, especially if the cleanup criteria is in the parts per billion range.

Waste characterization must be performed relative to the cleanup criteria. It is important to understand not only the nature of the contaminants but also, where a solid is to be treated, the structure of the solid and the binding of the waste to the solid.

Thermal destruction [35]: Thermal destruction is a mature technology employing a variety of combustion chambers, but in waste–site remediation applications, rotary kilns are most common. Thermal processes that destroy organic and inorganic contaminants by oxidation, pyrolysis, hydrogeneration, and reduction were considered.

Examples of technologies:

ALD Vacuum Technologies GmbH (Germany) [36]: commercialized technology used for mercury and cadmium recovery from batteries, lamps and other waste. The process, called vacuum thermal recycling (VTR), produces 99.99% - pure Hg and Cd directly. Mercury–bearing waste is heated to 300 ^{0}C–350 ^{0}C in a sealed oven at one millibar, vaporizing Hg, hydrocarbons and water. The vapors pass through an oxidation chamber, where hydrocarbons are converted to CO_2 and H_2O at temperatures of 800–900 ^{0}C. Finally, the vapors are condensed, yielding metallic Hg and water, which are separated. The first commercial plant has started in Lubeck, Germany, treating 200 Mg/year of mercury–laden waste and batteries.

Mercury Recovery Services Inc. (MRS process) [37]: In the process mercury-containing waste is mixed with relevant additives (patented, technology-specific) which decompose sulfur chlorides and sulfates. The mixture is then heated to approx. 95 ^{0}C to remove moisture and then to approx. 540–640^{0}C to vaporize the mercury. Mercury is recovered in a metallic form in the condenser. Some slight modifications of the methods may occur, depending on the type of waste to be remediated. In a U.S. copper smelter case the MSR process is reducing the mercury content of blowdown sludge from a sulfuric acid plant from 1,000-2,500 ppm down to less than 10 ppm. At a U.K. chlor–alkali plant, it has cut the mercury level in waste from 20% to 50 ppm.

Mobile Low-Temperature Thermal Treatment Process [38]: In 1990, Rurrkohle Umwelttechnik GmbH (RUT Germany) initiated development of a mobile low-temperature treatment system for a

range of soil contaminants, such as volatile hydrocarbons, chlorinated solvents, and mercury. The concept of commercial plants to treat mercury-contaminated soil follows:

- Prior to continuous feeding to the thermal treatment unit, contaminated soil or rubble is freed from any nibs or metallic fraction.

- In the evaporator, the contaminants are evaporated by indirect heating and vapor stripping. Then, the treated soil is cooled and remoistened with purified, processed water. The contaminated vapors are de-dusted with a cloth filter.

- The heat gradient required for the thermal treatment of the contaminated material is created in a thermo-oil heater. This unit causes the fuel oil tank and the facilities for the intermediate storage, feeding, and emptying of the heat transfer medium.

- The process steam plant is where the stripping steam is raised and superheated. The unit also contains facilities for the softening, degassing, and preheating of the feed water.

- Condensation involves a multi-step cooling of the hot, de-dusted vapors from the evaporator. The residual gas is finally purified with the help of activated carbon and then blown off. The contaminated residues, a mixture of water and mercury, are separated by gravimetric settling. The mercury is salvaged, and the water is cooled and used in the condensation process as quench water. Any excess water is fed to the process water treatment unit.

- The resulting process water contains mercury-both in dispersed and ionic forms. Process water treatment ensures physical and chemical purification of the water to meat the required effluent standards. The purified water is used for moistening the cleaned soil. Excess water is discharged.

On a pilot-scale, at the temperature 280^0C-320^0C, initial mercury levels of 300 to 11,000 ppm were reduced to 5 to 85 ppm.

According to RUT, the cost of soil cleaning using a full-scale plant based on this process would be about 90-160 U.S dollars per metric ton, depending on the contaminants. The total cost depends on the plant equipment required and waste disposal costs.

Soil washing/soil flushing

Soil washing [39]: An ex situ process employing chemical and physical extraction and separation techniques to remove a broad range of organic, inorganic and radioactive contaminants from soils. The process entails excavation of the contaminated soil, mechanical screening to remove various oversized materials, a separation process to generate coarse-and fine-grained fractions, treatment of those fractions (soil washing) and management of the generated residuals. By concentrating the contaminants in a smaller volume for further treatment, it enables more overall cost-effective treatment.

Soil washing may be used to treat soils containing a wide variety of contaminants including heavy metals.

Soil washing systems usually consist of the following six distinct process units:

- Pretreatment,
- Separation,
- coarse-grained treatment,
- fine-grained treatment;
- process washwater treatment, and
- residuals management.

Examples of technologies *application* [39]:

Toronto Harbour Commission's Soil Recycling Demonstration Project, Toronto, Ontario, Canada. 1-9. 1992

Volume: 4,400 tons

Key Contaminants: Cd, As, Cu, Pb, Hg, Zn, Ni, Oil and Grease, PAH's.

Treatment processes: Soil washing, metal extraction by chelation and organic reduction by aerobic bioremediation in upflow air reactors

The objective was to treat the soil to the extent that the soil can be reused on industrial land and metals removed can be recycled.

The soil wash system effectively cleaned coarse (> 6mm) and intermediate streams (0.063 to 6mm) to industrial standards.

Metal extraction processes can remove metals to meet residential and agricultural standards. Bioremediation processes can reduce oil and grease to industrial levels.

Zanesville Well Field, OH
Soil washing (preceded by vacuum extraction)
Media: soil
Contaminant: Metals (Pb, Hg)

Heijmans Milieutechniek b.v. Rosmalen, The Netherlands
Applied operations:-particle sizing, scrubbing with detergents and oxidants, flocculation, precipitation.
Pollutants treated: cyanides, heavy metals, PCAs, mineral oil, kerosene.
Rated throughput:-11 ton/hr.
Capital costs:-4.5 million dollars.

HWZ Bodemsan-ering Amersfoort, The Netherlands
Applied operations: particle sizing, scrubbing with detergents, flocculation, pH adjustment, carbon filters.
Pollutants treated: cyanides, heavy metals, aromatics, solvents, Cl-HCs.
Rated throughput: 22 ton/hr.
Capital costs: 3 million dollars.

Heidemij Realisatie Arnhem, The Netherlands
Applied operations: particle sizing, froth flotation with cleaning agents, washing.
Pollutants treated: cyanides, heavy metals, PCAs, Oils, Cl -HCs, Pesticides.
Rated throughput: 30 ton/hr.
Capital costs: 3 million dollars.
In-Pulp Decontamination of Soils, Sludges, and Sediments, United Kingdom [38]: This process combines conventional *ex situ* soil washing techniques with an innovative chemical treatment stage. "In-Pulp" processing, used alone or in combination with other physical processing techniques, offers an option to treat soil chemically in order to achieve low absolute values of metals. If a leachability standard is required, a chemical treatment may be attractive provided that the leaching

agents are more severe than the standard requires. Thus, applicability of the technology would depend on its ability to achieve regulatory requirements. A major advantage expected for this approach is the ability to remove contaminants from fine-sized soil fractions such as silt and clay. Two techniques are used to extract contaminants:

- leaching using acidic or alkaline reagents followed by absorption to activated carbon or ion exchange resin,
- adsorption by activated carbon or cation exchange resins in direct contact with a soil slurry.

Contaminants may then be desorbed from the recovered activated carbon or ion exchange resin, which is subsequently recycled.

Test materials consisted of contaminated soils and sediments from chlor-alkali and gas-metering sites and sites with mercury contamination. For the mercury-contaminated soil, oxidative and complexing conditions such as nitric acid, hydrochloric acid, and sodium hypochloride were used. To improve extraction of mercury, the approach included size separation to remove fine particles and was operated at higher temperatures. Ion exchange resins developed for mercury adsorbed the metal slowly. As a result of difficulties with this approach, a thermal option was investigated. Preliminary tests revealed that by heating contaminated materials to around 800^0C, treated soils achieved regulatory targets for mercury.

Harbauer Treatment Technology, Germany [38]: The Harbauer treatment system consists of two main processes: soil washing and vacuum distillation. The principle of this process is to concentrate the mercury in the fine-grained portion of the soil by soil washing and cleaning resulting in highly-contaminated, fine-particle fractions using vacuum distillation (a thermal evaporation process) as downstream treatment.

The fine-grained, highly-contaminated fraction of soil that is separated in the soil washing process is transferred to the vacuum-distillation process. This technology involves heating the soil to a temperature high enough to volatilize mercury. The Harbauer vacuum-distillation process heats the soil to temperatures between 350^0C-450^0C at a pressure of 50-150 hPa. The low-pressure conditions reduce the boiling points of the contaminants, resulting in lower energy consumption by the entire system and small flow of process gas amounting to only 3-5% of the gas flow, which is

usual for incineration plants. Thus, process gas treatment systems with much smaller capacities are needed, resulting in significantly lower investment and operational costs. Furthermore, vacuum distillation is a low-oxygen process that is assumed to avoid the generation of dioxins or other unwanted oxidation products. The relatively moderate heating temperature is considered to cause no severe changes to the mineral structure of treated soils. Initial mercury levels of 500 to 5,000 ppm were reduced to 50 ppm.

The estimated cost is approximately 320 U.S. dollars per metric ton.

Soil flushing [39]: An *in situ* process that uses water, enhanced water or gaseous mixtures to accelerate the mobilization of contaminants from a contaminated soil for recovery and treatment. Flushing solutions may include water, dilute acids and bases, complexing and chelating agents, reducing agents, solvents or surfactants. Surfactants can be added to increase the mobility of certain semi-volatile and inorganic contaminants, and chelating agents can be added to solubilize heavy metals.

Soil flushing accelerates a number of geochemical dissolution reactions that alter contaminant concentrations in groundwater systems, such as:

- adsorption/desorption,
- acid/base reactions,
- solution/precipitation reactions,
- oxidation/reduction reactions,
- ion pairing or complexation, and
- biodegradation.

Extraction methods

Solvent/chemical extraction [40]-is an *ex situ* separation and concentration process in which a non-aqueous liquid reagent is used to remove organic and/or inorganic contaminants from wastes, soils, sediments, sludges or water.

Examples of technologies

Oak Ridge National Laboratory (ORNL), Oak Ridge, TN. Researchers at ORNL are treating Hg–contaminated Department of Energy mixed waste using an advanced leaching process that is able to remove solid Hg species [41]. The process includes both the removal of Hg from the solid mixed waste and regenerating and recycling the leaching solution. Hg removal as high as 99.8% is achieved by contacting the contaminated solids with a potassium–iodide leaching solution at ambient temperature for between 2–4 h. The I_2 in the leach solution serves as an oxidant while the iodide ions serve as a complexant for Hg in the 2+ state.

During the leaching stage, a soluble Hg complex is formed that remains in the liquid phase as the leaching solution is separated from solids. These solids are then washed twice to remove trace leaching solution prior to discharge from the system. The leaching solution then is recycled using a multi–stage process.

In the first stage, spent leaching solution is contacted with steel wool to remove the Hg–complexes from the solutions as Hg metal. The steel wool is used for multiple cycles. Hg may be recovered from the steel wool and further processed in a full scale application of the technology. After the Hg–complex is removed from the leach solution, residual metal ions in the solution are removed by CaO precipitation. Finally, I_2 is recovered from a portion of spent solution to be used in the treatment of the next batch of Hg-contaminated solid mixed waste by adding H_20_2 to the solution.

GE Research & Development worked out a new process, commercialized by Metcalf & Eddy, Inc., which targets mercury over other metallic contaminants in a variety of tainted waste [42]. The closed–loop process incorporates three basic steps:

- reagent–based extraction,
- reduction and removal of extracted mercury, and
- regeneration and recycling of the extraction reagent.

During mercury extraction, which is carried out in agitated vessels or open heap–leach piles, the impacted materials are mixed with an aqueous, halide-based extraction reagent (patented). The extracted mercury remains in the aqueous phase, which in sent on the next step of the process,

while the leached soil or sediments are d-watered, rinsed and either backfilled or disposed of as a non-hazardous waste.

In one case, initial mercury levels of 3,700–6,000 ppm were reduced to less 10 ppm, after a single stage extraction. In another, mercury levels were reduced from 1,000-2,900 ppm to below 8 ppm.

Electrochemical remediation

Segall et al. [43] demonstrated that it is possible to remove heavy metals from a polluted site by using electrochemical methods. Since that time other scientists have also confirmed the possibility of using current to decontaminate sites polluted with heavy metals. These methods are not simple systems because they involve a number of phases: soil solution, air and solid phase. In addition high non-homogeneity of the soil further complicates the technology.

During the electrochemical process heavy metals are transferred to the pore water in a dissolved form or attached to colloids and move within the applied electric field. The method is found to be useful in many soil types, but its strength resides in fine-grained soils. This technology is valuable because it is in fine-grained soils that other remediation methods fail [43, 44, 45, 46, 47].

Since mercury species can be bound in micropores, diffusion limits the speed of extraction processes to a great extent, but the applied electric field reduces the problem of mass transport in the in the remediation process. Three transport phenomena are responsible for electro-kinetic mercury movements in soil [44, 45]:

- electro-migration, where ionic species are transported to the electrodes;
- electro-osmosis, a flow of the pore water can result in a transport of charged as well as uncharged species present in the pore liquid towards the cathode; and
- electrophoresis, movement of collide particles against a fixed dispertion center-katarophase or anarophase.

Cox et al. [45] compared the cleaning of an artificially contaminated loam soil with a field-contaminated sandy loam soil in a bench-scale cell using an iodine/iodide lixivalent for controlling the redox potential. The authors showed that the height of the oxidation potential is essential for the high process efficiency. When iodine was added, up to 99% of the mercury in the synthetic

contaminated soil could be removed. In contrast to their good results, only 6% of the mercury in the soil originating from the contaminated site could be removed. They assumed that the organic material present in the soil, which consumed oxidant iodine, was responsible for this low level of removal.

Hansen et al. [46] investigated soil from a contaminated site using an electrodialytic remediation technique (EDR) - ion-exchange membranes. Initial mercury concentration was 685 ppm and the removal rate was up to 55% (with an average of 23%), but the real removal potential was not clear since the duration of the experiments was not varied.

Thoeming et al. [44] carried out four electrodialytic remediation experiments on the mercury-polluted soil: two experiments of 27 days duration and two experiments of 54 days duration. The experimental set-up used was the same in all experiments. The authors showed that during the electrodialytic treatment an increase of the content of non-metallic mercury occurred and a corresponding decrease of the content of elemental mercury which indicates a transformation of the latter species into any other non-metallic species. Generally, oxidation of mercury by dissolved oxygen in a solution is kinetically inhibited and quite slow.

Sobra and Thoeming [47] defined safe conditions for treating high mercury content residues (≥ 6 g/kg of residue), from the chlor-alkali plant, by an electrolytic process. This process makes use of the conventional aqueous sodium chloride electrolysis for generating oxidant species and then dissolving elemental mercury, in the bulk of the reaction system. The soluble mercury species ($HgCl_4^{-2}$) generated are simultaneously deposited at the graphite cathode surface making the decontamination feasible. This study also incorporates a thermodynamic approach of the process as a whole and, additionally, the influence of some process parameters such as sodium chloride concentration, pH, and current densities.

Thoeming and Franke [48] developed the electroleaching method. Electroleaching offers a cheaper alternative which is a hydrometallurgical process combining wet extraction and electrolytical preparation of the leachate. There are two advantages of such a process compared with retorting processes. Because of far larger thermodynamic separation factors the capital costs are much lower. During electrolysis the redox potential of the chloride solution increases dramatically.

Consequently this solution is capable of oxidizing Hg^0 and Hg^{+1}compounds in soils readily to Hg^{+2}. The extract this species forms is a strong and soluble complex - $HgCl_4^{-2}$. The mercury-loaded aqueous stream is treated again electrolytically- mercury is deposited cathodically and reagent is regenerated. Further optimizations of the process with regard to minimization of toxicity are needed.

Bioremediation and phytoremediation

Bioremediation [49] exploits the ability of certain microorganisms- heterotrophic bacteria and fungi- to degrade hazardous organic materials to innocuous materials such as carbon oxide, methane water, inorganic salts, and biomass. Microorganisms may derive the carbon and energy required for growth through the bioremediation of organic contaminants or through the transformation of more complex, synthetic chemicals through fortuitous co-metabolism. These fortuitous reactions stem from the broad substrate specificity of some microbial enzymes. (This type of transformation is termed „microbial metabolism of contaminants that are not growth substrates"). A variety of enzymatic reactions: oxidation, hydrolysis, reduction, dehalogenation, and reduction of nitro groups- catalyze co-metabolic processes.

The indigenous microbial community may not have the capability to degrade specific synthetic chemicals of concern at a particular site. If treatability studies show no degradation (or an extended delay before significant degradation is achieved), inoculation with strains known to be capable of degrading the contaminant may be helpful. In a process known as bioaugmentation, microbial strains are added that cannot use the contaminant as a growth substrate but, nevertheless, completely degrade the contaminant: a novel strain of *Pseudomonas cepecia* has been used to degrade trichloroethylene; *Phanerochaete chrysosporium* biodegrades a wide range of organic compounds with nonspecific extracellular peroxidases. Pentachlorophenol has been treated in soil bioreactors by adding active biomass that has been grown on another substrate.

Microorganisms can catalyze a wide range of oxidation, reduction and methylation reactions involving metals. These reactions can results in mobilization, immobilization or volatilization of the metals. Such reactions are well-documented and show considerable potential but have been little used in bioremediaiton because they do not destroy the metals.

The primary importance of metals in bioremediation lies in their toxicity to microorganisms. Heavy metals are used as biocides and can inhibit or kill the bacteria used in biotreatment. Therefore, if they are present at toxic levels in mixed wastes they must be removed or their toxicity will reduce the waste to the toxic levels found prior to bioremediation.

In recent years it was stated that the bacteria type *mer*$^{+}$ could be used for the bioremediation of mercury-contaminated soil [17, 509]. *Mer*$^{+}$ bacteria convert organic and ionic mercury compounds to the volatile and less toxic elemental form. Hg^{0} rapidly evaporates through cell membranes and walls.

Example of technology

Wagner-Doebler et al. [51] developed mercury removal from chemical wastewater by microoganisms on a technical scale. The enzymatic reduction of Hg^{+2} to water insoluble Hg^{0} by mercury resistant bacteria has been used. They have used seven types of bacterial *Psesodomonos*: 4 subspecies of *Psesodomonos putida*, 2 subspecies of *Psesodomonos stutzeri*, and 1 subspecies of *Psesodomonos fulva* immobilized on carrier material inside a 700 L packed bed bioreactor.

Incoming wastewater at pH 2-3 was neutralized to pH 7.0± 0.5, supplemented with a small amount of nutrients to provide energy to the bacteria, and then run through the packed bed bioreactor containing the mercury-resistant bacterial catalysts as a biofilm on the carrier material. The effluent from the bioreactor was passed through an activated carbon filter to remove remaining traces of mercury. The bioreactor inflow valve automatically closed and opened the bypass if wastewater inflow parameters lay outside of preset ranges, namely a pH value above 7.5 or below 6.5; mercury inflow concentration above 10 mg/l; a temperature above 47^{0}C; or chlorine concentrations above 0.5 mg/L. Treated or bypassed wastewater re-entered the factory wastewater treatment system. Conductivity, redox potential, and oxygen concentration, as well as mercury concentrations in the wastewater inflow, at the bioreactor outflow, and in the activated carbon filter outflow were determined continuously. Removal of 90-97% of mercury from chlor-alkali wastewater resulted.

This technology offers a highly efficient way to extract mercury from polluted wastewater. It is environmentally friendly, since it works at ambient temperature, requires little electric energy, and

no additional chemicals. Operating costs are low. For 100 m^3 of wastewater cleaned, about 15 U.S. dollars are required for nutrients to feed the bacteria.

Technology prognosis: New bioremediation approaches -use of vegetation– phytoremediation methods. Phytoremediation of metal-contaminated soils offers a lower cost method for soil remediation. Some extracted metals may be recycled for value.

Phytoremediation encompasses several mechanisms, including [17, 49, 50]:

- phytoextraction or phytoconcentration, where contaminants are concentrated in the roots, stem and foliage of the plant;
- phytodegradation, where plant enzymes help catalyze the breakdown of the contaminants;
- rhizosphere biodegradation, where plant roots release nutrients to microorganisms which are active in biodegradation of the contaminants;
- volatilization, where transpiration of organics, selenium and mercury run through the leaves of the plant; and
- stabilization, where the plant converts contaminants into a form which is not bioavailable, or the plant prevents the spreading of a contaminant plume.

Meagher [52, 53] engineered several plant species, for example: *Arabidopsis*, tobacco, canola, yellow poplar, rice, to express the bacterial genes, *merB* and/or *merA*, under the control of plant regulatory sequences. These transgenic plants acquired remarkable properties for mercury remediation.

Summary of the literature review

The literature review focused on the technologies for the remediation of soil contaminated with mercury revealed that the applied technologies have a number of significant drawbacks such as:

- high capital costs,
- they prove effective only at extremely high Hg concentrations,
- most of these technologies have been applied for waste, water and sludge remediation rather then soil,

- the technologies are complex, usually multiple phases and require preparation and pre-treatment of the cleaned-up media,
- several remediation techniques have already been applied, emerging in the laboratory scale, pilot-scale or just invented, and
- taking into account the investment costs, it was stated that beside phytoremediation, the largest potential for full scale applications represent these methods which are based on mercury stabilization in soil.

It can be concluded that none of the studied and presented in this report technologies has been identified to be effectively applied to cleanup soils contaminated with mercury. According to the latest literature data, the current approaches to soil remediaton from heavy metals focus on the use of plants and microorganisms as these methods are cost effective, environmentally friendly. The data from the up-to-date laboratory experiments is promising and seems to prove the applicability of phytoremediation for soil clean-up. Attention however should be paid to the problem of safe handling of the contaminated crops.

Recently attention has been paid to the methods enhancing natural soil attenuation processes. The following processes can be categorized into this group: transformation of easily soluble compounds into insoluble compounds or mobile into immobile compounds.

IETU in cooperation with FSU undertook an effort to work out a technology for mercury stabilization in soil which would combine the activity of chemical compounds, microorganisms and plants to effectively confine the contaminant. This year's activities addressed the development of the technology at lab-scale.

Site Identification

The site in question is a chemical facility known as AZOTY, were mercury and its compounds are used in multiple manufacturing processes. The AZOTY facility in Tarnow has been operating for over 70 years becoming a well recognized chemical enterprise at home as well as international markets. The facility produces about 100 various products, among which the most important are

the following: caprolactam, polivinyl chloride (PVC), polyamides, polytroxane, fertilizers, cyanogen derivative compounds and chlorine products.

For several decades the AZOTY facility has been using mercury and its compounds in manufacturing processes. Since 1933 metallic mercury has been used for chlorine and sodium hydroxide production by electrolysis of salt solutions. During the electrolysis process gaseous chlorine is generated at the anode and mercury amalgamate is generated at the Hg cathode. Graphite anodes were used until the end of 1991. Since 1992 titanium and fixed parameter anodes have been applied. Gaseous chlorine is permanently supplied from the cell rooms and, after drying, is redirected to other production processes. Sodium amalgamate is transferred from the electrolytic tank to the decomposer in which, after the addition of water, it decomposes into soda lye – 45%, hydrogen and mercury. The generated hydrogen is utilized in the production of PVC plastic and hydrochloric acid. From the decomposer mercury is pumped to the electrolyser tank. In 1999 the chlorine production volume was approximately 29,000 Mg/yr.
Since 1961 the facility has been producing PVC from hydrochloric acid and acetylene in the presence of a catalyst – mercury chloride deposited on activated carbon. Until the end of 1991 this catalyst was produced in the AZOTY facility in the amount of 10-weight percent. In 1992 the facility began to import the catalyst from an Italian company „Aussimont Catallzzatorri". The company agreed to accept an equivalent volume of used catalyst. In 1999 the PVC production volume was on the level of 35,000 Mg/yr.

The AZOTY facility is located in the southeastern part of Poland, in the basin of the Dunajec River - a tributary of the Vistula River (Map 1). More specifically the facility is situated between the lower run of the Dunajec River and the mouth of the Biala Tarnowska River to the Dunajec River. The geological composition of the site consists of Quaternary and Tertiary formations. The Tertiary formations are basically Miocene silts and dusts – Grabowieckie layers and Chodonickie layers. The Tertiary sediments are covered with Quaternary clastic river formations. In the floor, coarse-grained gravel with cobbles transforms into fine-grained sands, clayey dusts and dusty clays as one moves upward in the soil profile. The clastic formations are tipped with clayey-sandy banks as well as loam.

Geology

The youngest formations recognized in the Tarnow Region are Tertiary and Quaternary sediments.

Tertiary sediments are formed as clays and silts of Miocene aged Grabowieckie and Chodenickie Layers. They probably cover older, Oligocene formations.
Natural clastic sediments of Quaternary age lay directly on Tertiary formations. The thickness of the clastic sediments is about 8.2-8.3 m.

The bottom part is formed by thick-grained gravel with pebbles, which transform upwards into fine-grained sands and loamy silt and silty loam.
Stratigraphically, the youngest layers are anthropogenic formations composed of loamy-sandy banks, with thickness varying between 2.0-3.4 m. Locally, aleurites can be diagnosed. with a maximum thickness of 1m.

Up to a maximum depth of 5 m in the soil profile, layers of organic, humus soil can be observed, while the older and deeper profile is composed of mineral soil. The table below presents the profiles of the boreholes [M-1and M-2] in areas most heavily contaminated with mercury.

Table 3. Boreholes profiles

M-1 (Mercury Regeneration Facility Site)	
The depth of bore-hole [m ppt]	**Lythology**
0.0 - 2.0	anthropogenic sediments (sand-gravel-loam mix, sandy silts + brick)
2.0 - 3.0	brown aleurite
3.0 - 3.4	brown loamy sand
3.4 - 4.5	loamy sand + fine grain gravel
4.5 - 5.6	dark yellow sand + gravel
5.6 - 7.2	gravel + pebbles + different grain sand
7.2 - 8.3	gravel + pebbles
8.3 - 9.3	gravel + grey silt
M-2 (Incineration site)	
0.0 - 3.4	anthropogenic sediments (silt + loam + slim + sand + brick)
3.4 - 4.2	yellow fine grain sand
4.2 - 6.0	grey sand + gravel + pebbles
6.0 - 8.2	gravel + pebbles (max ø 130 mm)
8.2 - 9.2	silty sand + grey silt

Hydrogeological conditions

The discussed area is located in the fork of the Biala and Dunajec River, which determine the groundwater regime of the region. Within the studied profile, one Quaternary groundwater level of free character was identified. At the facility site, it stabilizes at the depth of 187.95 (M-2) - 187.33 (M-1) m above the sea level. The flow of the Quarternary groundwater level is north-west, where it is drained by the Dunajec River, and toward north-east, where is drained by the Biala River. The predominate flow direction for the Biala and Dunajec Rivers' fork is north-west, where higher hydraulic depressions of the water table were identified. The aquifer is composed of sand-gravel-loam mix and gravels with pebbles, limited from the bottom by Tertiary clays of maximum permeability coefficient of 10.8×10^{-4} m/s. Below the variability of the permeability coefficient at M-1 and M-2 piezometer is presented.

Table 4. Variability of the infiltration coefficient and total mercury content in soil

Piezometer	The depth of soil sample [m u.g.l.]	Total Mercury content [mg/kg dry matter]	Effective diameter d_{50} [mm]	Permeability coefficient k_{10} [m/s]
M-1	0.5	131.8	1.200	51.4×10^{-6}
	1.0	27.63	0.030	3.2×10^{-6}
	1.5	3.193	0.026	2.4×10^{-6}
	2.0	8.082	0.042	6.3×10^{-6}
	2.5	0.324	0.053	10.0×10^{-6}
	3.0	0.398	0.035	4.4×10^{-6}
	4.0	0.528	0.550	10.8×10^{-4}
	5.0	0.283	0.024	2.1×10^{-6}
M-2	0.5	238.7	0.030	3.2×10^{-6}
	1.0	22.74	0.044	6.9×10^{-6}
	1.5	5.262	0.030	3.2×10^{-6}
	2.0	55.45	0.035	4.4×10^{-6}
	2.5	10.05	0.024	2.1×10^{-6}
	3.0	1.012	0.022	1.7×10^{-6}
	4.0	0.650	0.370	4.9×10^{-4}

Hydrochemical conditions

Variability of mercury content was determined based on the data obtained from Hg content measurements in soils and groundwater. Waters show Hg content variability in the range of 0.281 - 0.724 µg/L close to water table and 0.325 - 0.402 µg/L at the bottom part of the aquifer. An increase in mercury content has been observed towards the Biala River. The water of the Biala River also exhibits elevated mercury content in comparison with the Dunajec River water. This fact is probably related to the direction of groundwater flow. The Mercury Regeneration and Incineration Site facilities, which are potentially exposed to higher mercury content, are located at the eastern side of the watershed between the Biala and the Dunajec Rivers. Archival data and measurements made in 1999 prove that the watershed is not stable here. The watershed depends on the level of water in both rivers and on the season of the year. However, it has no impact on the change of the groundwater flow direction in the Mercury Regeneration Facility Site and Incineration Site. At these sites the groundwater is drained by the Biala River in the northeast.

Mercury content in the soil tends to decrease with depth; however, locally, the tendency may be disturbed at the depth of approx. 1.5 m u.g.l. below which Hg content increases. Moreover, two profile zones may be distinguished which significantly differ from each other. The border limit between them is at the depth of approx. 2 (2.5) m u.g.l., below which mercury content in soil abruptly decreases. In the upper section of the profile, mercury content varies in the range of 238.7 to 3.193 mg/kg of dry mass, while at the lower section mercury content was found in the range of 1.012 - 0.283 mg/kg of dry mass. It seems that this phenomenon should be correlated to the occurrence of insulating sediments of aleuritic character at the border limit.

Site Characterization

There are three sources of mercury emission to the atmosphere from the AZOTY Site: chlorine production using electrolysis, recovery of mercury from waste materials and coal combustion in the facility's power and heating plant. Annual mercury emission volumes from chlorine production are presented in Table 5 and Figure 2.

Table 5. Mercury emission volumes [kg/year]

	1991	**1992**	**1993**	**1994**	**1995**	**1996**	**1997**	**1998**	**1999**
Mercury	1754	218	198	202	172	163	203	91	55

Hg emission reduction between 1991 and 1992 was the result of changes in the technology :

- replacement of graphite anodes to fixed parameter titanium anodes,
- installation of carbon filters at alkaline exhausting gases outlet,
- recirculation of acid-exhausting gases.

Air emissions were evaluated by collecting data on mercury deposition at points located in and near the site (7 points, Map 1). During the period of 1995-1999 the deposition rates of mercury in the site and near the site are similar. Annual mercury deposition (median) rangess between 0.86 mg/m^2/year and 1.12 mg/m^2/year.

A single case of mercury deposition higher than the median was noted in 1995, at the point located in the vicinity of the main road. However, the available data was not sufficient to interpret such high deposition accordingly.

Changes in mercury deposition values from 1995-1999 are presented in Table 6 and Figure 3.

Table 6. Hg deposition [mg/m^2/year] at the AZOTY facility site and in its vicinity

Location and sampling point No on the map	**1995**	**1996**	**1997**	**1998**	**1999**
Sports House **5**	3.01	0.92	0.97	0.86	1.22
Main Wastewater Treatment plant **6**	1.08	0.65	0.96	0.72	1.12
Zbylitowska Góra **7**	1.02	0.78	0.90	1.12	1.16
Oxygen plant **8**	1.58	1.53	1.12	0.98	0.91
Pumping Station **9**	1.11	0.79	0.86	0.84	1.21
Biala **10**	0.80	0.92	0.98	0.88	0.91
Czajki **11**	0.90	1.10	0.95	0.81	0.98

The volumes of mercury deposition at the site and in its vicinity are similar and show no significant impact on mercury content in the soil. For example mercury content in soil at Zbylitowska Gora (point 7 - potable water intake point for Tarnow) amounts to 0.090 mg/kg of soil dry mass, while in the Main Wastewater Treatment Plant (point 6) values of 1.799 mg/kg of soil dry mass are found.

In order to determine surface water contamination, data from measurements carried out along the Dunajec and Biala Rivers were used. Annual median mercury concentrations in these rivers are presented in Table 7 (Figure 4 and 5). The permissible mercury concentration for Class I surface waters is 1.0 µg/L.

Table 7. Mercury concentrations [µg/L] in the Dunajec and Biala Rivers

Year	Dunajec		Biala	
	upstream from the facility 2	downstream from the facility 1	upstream from the facility 4	downstream from the facility 3
1991	0.5	0.6	0.8	3.9
1992	0.5	0.4	0.7	2.5
1993	0.5	0.6	0.6	1.7
1994	0.6	0.4	0.5	1.5
1995	0.2	0.5	0.6	1.1
1996	0.3	0.3	0.5	0.5
1997	0.5	0.2	0.5	0.4
1998	0.1	0.3	0.3	0.4
1999	0.3	0.25	0.1	0.3

Based on the data, the Dunajec River water has not exceeded permissible standards at any of the measurement points during the 10 year study period. In the period of 1991-1995, the measurement points downstream of the facility on the Biala River, exceeded permissible mercury standards for water purity Class I.

No mercury was detected in water collected from piezometers installed in the vicinity of the AZOTY facility in the years 1995-1999. However, water collected from piezometers installed within the facility during 1997-1999, near the sites contaminated with mercury (mercury regeneration facility and incineration facility), reveal concentrations in the range of 0.21 to 3.9 µg/L. Some of these concentrations exceeded the permissible groundwater standard of 2 µg/L in Poland. Mercury concentration in water collected form piezometers installed in the vicinity of the mercury-contaminated sites are presented in Table 8.

Table 8. Mercury concentration [μg/L] in groundwater

Year	M - 1	M - 2	M - 3	B - 1
1997	0.46	0.33	0.56	0.44
1998	3.6	3.0	3.9	3.4
1999	3.0	3.1	3.0	2.9
2000	2.5	2.9	2.6	2.1

Data analysis from the long term environmental monitoring programs indicate that the area in the vicinity of the AZOTY facility has not been contaminated with mercury. The volume of the annual emissions, mercury deposition and data from the analysis of surface water (Dunajec and Biala Rivers) indicate that mercury-contaminated soils at the AZOTY facility site do not cause contamination of surface and ground waters. Thus, the contaminated area is enclosed within the facility site.

Mercury contents in soil

On the basis of the investigation of mercury content in soil at the AZOTY facility carried out in the period of 1994-1995 and in 2000, four mercury-contaminated sites were identified:

- incineration site, at which discarded chemical equipment was disposed and anticorrosive coatings were burnt out and incinerated in an uncontrolled way (approx. 15 000 m^2) - Maps 2 and 3,
- site in the vicinity of the two cell rooms for chlorine production (approx. 2500 m^2) Maps 5 and 6,
- site in the vicinity of installation for mercury-containing waste regeneration (approx. 300 m^2), and
- site in the vicinity of vinyl chloride and PVC production installation (approx. 1000 m^2).

Soil was sampled for analysis from the following depths:

- top layer 0-20 cm,
- 40-60 cm depth layer,
- 90-110 cm depth layer,
- 140-160 cm depth layer.

The results of the total mercury concentrations (median) in individual levels and four sites of mercury contamination are presented in Table 9 and Figure 6.

Table 9. Total mercury contents in contaminated soil [mg/kg of dry matter] in vertical cross section.

Depth (cm)	Electrolysis Cells	Mercury regeneration facility	PVC - production facility	Incineration site
0-20	40.0	557.0	261.6	161.5
40-60	9.83	12.23	9.13	37.86
90-110	4.95	18.55	2.23	12.18
140-160	2.60	3.28	0.93	4.90

This demonstrates that mercury concentrations in soil near individual technological installations show high variability in all four depths. The highest mercury concentrations were found in the surface layer adjacent to the regeneration facility, whereas the highest concentration of that metal in the 40-60 cm depth was observed in the vicinity of the incineration site. In all sites, reduction of mean mercury concentrations decreases with increased depth (Figure 6).

Total mercury concentrations in soil from points located in and near to the site (6 points, Map 1) was determined. Changes in mercury contents at these points are presented in Table 10.

Table 10. Total mercury contents in soil [mg/kg of dry matter] in vertical cross section

Depth (cm)	Main Wastewater Treatment Plant - 6	Zbylitowska Gora - 7	Oxygen Plant - 8	Pumping Station - 9	Biala - 10	Czajki - 11
0-20	1.799	0.090	1.410	1.016	0.519	0.657
40-60	0.951	0.072	0.945	0.723	0.234	0.287
90-110	0.730	0.031	0.561	0.415	0.122	0.176
140-150	0.559	0.019	0.358	0.323	0.095	0.112

In all points, the reduction of mercury contents decreases with the increase in depth. The lowest mercury concentration was identified in Zbylitowska Gora-potable water intake point for Tarnow.

The assessment of mercury contamination for various elements of the environment at the AZOTY site and its vicinity demonstrated:

* reduction of atmospheric mercury emission by nearly 30 times in the last decade,
* similar levels of magnitude of mercury deposition at all measurement points,
* mercury content in the upper and lower run of the Dunajec River does not exceed permissible levels for Class I waters,
* reduction of mercury content in the lower run of the Biala River by 13 times in the last five years,
* no mercury content was identified in the groundwater in the vicinity of the facility.

However, the vertical distribution of mercury concentrations in soil and Hg concentrations in the piezometers samples indicate the vertical migration of mercury. This justifies the development of a technology to immobilize mercury and its compounds. At present, research is focused on the selection of chemical substances and plants that would effectively bind/stabilize mercury and its compounds in soil.

Soil physical and chemical properties

Approximately 800 kg of soil were collected from the surface (0-50 cm depth) of the two potential fields (mercury regeneration facility and incineration facility) for use in mercury stabilization studies. Samples were collected from different areas within the selected field and combined into one sample. This soil sample was analyzed physically and chemically. Based on the mechanical composition of the soil sample, it was categorized as a light clay. Table 11 presents the data on mechanical soil composition.

Table 11. Mechanical composition of soil (n = 5)

Soil fraction	Unit	Range	Mean	SD
Skeletal > 2 mm	%	28.3 - 32.8	29.6	± 2.44
Sand 2.0-0.1 mm	%	41.2 - 45.3	43.1	± 2.00
Dust 0.1-0.02 mm	%	21.4 -25.2	22.9	± 2.10
Floatable < 0.02 mm	%	34.1 - 35.2	34.4	± 0.61

The data from the soil physical and chemical analyses are presented in Table 12.

Table 12. Data from the analysis of physical and chemical properties of the soil (n = 5).

Investigated component	Unit	Range	Mean	SD
Organic carbon	%	1.61 - 1.86	1.77	± 0.14
Organic matter	%	2.77 - 3.21	3.05	± 0.24
$CaCO_3$	%	0.48 - 0.52	0.50	± 0.02
pH in H_2O		7.69 - 7.71	7.70	± 0.01
pH in KCl		6.90 - 6.93	6.92	± 0.015
EC	µS/cm	199.9 - 202.9	201.5	± 1.51
CEC	cmol +/kg	14.2 - 16.8	15.27	± 1.36
Hydrolytic acidity	mval/100g	2.1	2.1	
Total Nitrogen	%	0.104 - 0.105	0.105	± 0.0006
P_2O_5	mg/100g	0.13 - 0.14	0.137	± 0.005
K_2O	mg/100g	12.1 - 12.3	12.2	± 0.115
Ca	mg/kg	2644 - 3 210	2883	± 293
Mg	mg/kg	108 - 140	123	± 11.2
K	mg/kg	209 - 217	212	± 4.62
Na	mg/kg	30 - 40	36.7	± 4.34

Mercury speciation

A correct assessment of the selection of stabilizing agents (chemical compounds, plant material, microorganisms) or mercury-binding compounds in the soil requires not only defining its total contents but also the knowledge of the occurrence of mercury species in the soil.

According to professional literature the most frequently determined forms of occurrence of heavy metals (Pb, Cd, Zn, Cr) in the soil include the following forms and substances allowing for their extraction:

- water soluble - extractant reagent - deionized water,
- exchangeable - extractant reagents - 0.05-0.1M $CaCl_2$, 0.1M $HN0_3$, 1M NH_4OAc, 0.1M $Ca(NO_3)_2$, 0.1M $NaNO_3$
- sorbed and organically bound - extractant reagent - 0.5M HOAc,
- bound, occluded in oxide and secondary clay minerals - extractant reagents - 1M HNO_3, 0.05M EDTA, acid $(NH_4)_2Ox$, 0.005M DTPA,
- residual; primary mineral lattice - extractant reagents - HNO_3/HF, HNO_3/HCl.

Most of the extractants are not specific to an individual phase or to a particular elemental form. Speciation in this narrowly defined sense is a difficult task, because most extraction procedures will themselves change the speciation.

In the case of mercury there are many reports in the literature concerning the determination of the mercury organic compounds content, particularly CH_3Hg^+ in the soil. The mercury organic form content in the soil varies from 0.1% to 2% for soils of organic substance content of several percent. In the case of soils where the content is from several to several tens percent organic substances the content of methylmercury may reach up to 10%. There are few reports on the determination of mercury species other than organic compounds.

In the case of Hg, even the weakest complexes are so strong that they have to be considered unavailable according to the classical definition. However, the analogy to other heavy metals can be kept up by considering complexes with hard ligands, such as oxygen- containing a surface group

on clay minerals, oxides, and hydroxides as weak, potentially reversible and thus available. The group of Hg species also includes intact compounds that are water-soluble under natural pH and salinity conditions, e.g. $HgCl_2$ or complexes with water-soluble organic matter. This group of Hg species will also be called mobile here because the ionic or covalent species set free from solid phase are available for aqueous phase transport and transformation. The terms unavailable and immobile used for compounds in which the mercury-binding is either chemically irreversible under natural conditions or which, like HgS, are so insoluble in water that they do not liberate Hg ions. Mercury in those complexes is bound to soft sulfur containing ligands and their environmental mobility, consequently, is very low.

According to Dmitrow et al. (53), in order to help characterize the geochemistry of mercury in terrestrial and aquatic systems, a sequential extraction procedure was devised and applied to determine the partitioning of Hg between three operationally-defined solid compartments: organic matter, reactive Fe and Mn oxyhydroxides, and a residual clay and sulfide fraction. Total mercury was determined following digestion/reaction with a 10:1 nitric:hydrochloric acid solution. The proposed mercury extraction procedure consists of three steps:

- association with organic matter - extraction with 1M NaOH,
- association with reactive Fe and Mn - extraction with 1M HCl,
- association with solid residue - extraction with HNO_3/HCl solution (10 :1).

According to Windmoller et al. (22), thermal release analysis of mercury species in contaminated soils was performed by temperature-controlled continuous heating of samples in a furnace coupled with an atomic absorption spectrometer. It was shown that this method allows for the identification of the different redox states of Hg species through their characteristic releasing temperature ranges. The method was applied to Hg contaminated from an inactive chlor-alkali production plant in former East Germany, and from a gold mining area in Brazil. Analysis of original samples revealed that the mercury does not remain as Hg^0 in either matrix, and that oxidation of mercury occurs in both cases. In the German matrix this oxidation occurs to a greater extent. Samples with high amounts of total mercury (up to 465 ppm) do not show the presence of Hg^0, but only Hg^{2+}, likely bound to humic substances.

Wallschlager et al. (54) proposed a mercury-specific sequential extraction procedure. based on the philosophy of solubilizing individual, intact relevant mercury species rather than sequentially destroying the entire matrix. Less drastic extraction conditions have been chosen, which simultaneously provide some hints to the (re)mobilization behavior of Hg under natural conditions. The proposed mercury sequential extraction procedure consists of five steps:

- neutral extraction with deionized water,
- acid extraction with 0.01M HNO_3,
- alkaline extraction with 1M KOH, acidification to pH 2 with HNO_3,
- sulfide extraction with a solution of $Na_2S{\cdot}9\ H_2O$,
- oxidative extraction with concentrated HNO_3.

Schwesig et al. (45) determined total mercury, mercury fraction and methylmercury. The authors used the following sequential extraction procedure:

- water soluble extraction with deionized water,
- exchangeable extraction with 1M NH_4OAc,
- humic/fulvic fraction extraction with 1M NH_4OH and subsequent acidification to pH 1 with HCl,
- residual was calculated as the difference between the sum of these fractions and total mercury.

The literature review revealed a lack of unified analytical procedures for sequential speciation of mercury forms occurring in soil. To realize the project activities, aside from the determination of total mercury content, the following sequential extraction procedure for the determination of mercury species was applied:

- water soluble fraction extraction with deionized distilled water,
- exchangeable fraction extraction with1M NH_4OAc,
- humic/fulvic fraction extraction with 1M KOH and subsequent acidification to pH 1 with HCl,
- organic/sulfide fraction extraction with 0.1 MHNO_3 and H_2O_2,
- residual digestion with aqua regia.

A 20 g sample of material was used for the extraction. The sequential extraction procedure (SEP) was carried out as follows:

- shaking the sample material with 100 mL of deionized-distilled water followed by centrifugation (water soluble fraction),
- shaking the residue with 100 ml of 1M ammonium acetate for 1h and centrifugation (exchangeable fraction),
- shaking the residue with 100 mL 1M KOH for 1h, centrifugation, digestion of the aliquot of solution with 20 mL of concentrated HCl and 20 mL of H_2O_2 for 2h at 85^0C (humic/fulvic fraction),
- adding 25 mL 0.1M. HNO_3 to the residue, heating for 2h at 85^0C, addition of 40 mL H_2O_2, heating for 1h at 85^0C, addition of 100 mL of 1M NH_4AC/HNO_3 (6%) solution, shaking for 1h, and centrifugation (organic/sulfide fraction),
- 1.0 g of the residue with 10 mL aqua regia digest by microwave.

The mercury content in all fractions was determined by cold vapor atomic absorption spectrometry (CVAAS) after reduction of Hg^{+2} to Hg^0 with $SnCl_2$ solution. Total mercury content was determined by digesting 1.0g of the fresh sample in aqua regia according to the method for the residual fraction.

Sequential speciation analysis of mercury species in soil was carried out. The obtained data are presented in Table 13.

Table 13. Data from the sequential extraction of mercury forms in soil [mg/kg dry matter] (n=10)

Mercury forms	Range	Mean	SD	RDS [%]
Water soluble	0.363 - 0.567	0.437	0.082	18.77
Exchangeable	2.405 - 3.795	3.092	0.61	19.74
Fulvic and humic bound	27.5 - 40.66	33.44	4.19	12.53
Organic/sulfide bound	95.88 - 119.2	106.1	9.58	9.03
Residual	98.76 - 128.6	110.4	9.79	8.86

The mean total mercury content in the analyzed soil sample was 261.5 ± 9.21 mg/kg (dry matter) with a range of 250.3-273.7 mg/kg (dry matter).

The percentage of Individual mercury species in total Hg in soil was as follows:

- mercury compounds easily soluble in water - 0.17%,
- mercury compounds exchangeable - 1.18%,

- mercury compounds bound with fulvic and humic acid - 12.78%,
- mercury compounds organic/sulfide bound - 40,60%,
- mercury compounds bound with residual - 42.22%.

Anions in soil were determined. Mean concentration of anions in soil:

- F^- - 7.96 ± 0.7 mg/kg dry matter, (range 7.05-8.95),
- Cl^- - 12.38 ± 0.79 mg/kg dry matter, (range 11 -13.45),
- NO_3^- - 25.88 ± 3.04 mg/kg dry matter, (range 21.8 -30.1),
- SO_4^{2-} - 88.35 ± 7.69 mg/kg dry matter, (range 79.7-103.4).

Binding compounds and plant species were selected carefully with consideration of physical and chemical properties of the soil as well as mercury concentration. The key goal was to prevent undesired soil pH changes, which could lead to the release of mercury.

MERCURY STABILIZATION IN SOIL USING CHEMICALS

The goal of this subtask was to select appropriate chemical substances which would stabilize or bind mercury and its compounds occurring in the contaminated soil in such a way as to enable their off-site migration to other environmental compounds, especially groundwater.

In order to achieve the objective of the subtask labscale experiments were carried out to identify a chemical substance which most effectively binds or stabilizes mercury and its compounds in soil as well as to determine changes in mercury content (its individual species) in soil as a function of time. The following chemical substances were and continue to be tested:

- fine sulfur,
- granular sulfur,
- zeolites,
- mixture of dolomite and zeolite (10 : 1) with an addition of brown coal.

The experimental plan was as follows:

1) Hg-contaminated soil blank (3 replications),
2) Hg-contaminated soil + fine sulfur in the amount 0.5%, 1% and 5% (3 replications),
3) Hg-contaminated soil + granular sulfur in the amount 1%, 5% and 10% (3 replications),
4) Hg-contaminated soil + zeolite in the amount 1%, 5% and 10% (3 replications), and

5) Hg-contaminated soil + mixture of dolomite and zeolite with brown coal in the amount 1%, 5% and 10% (3 replications).

* soil sampling from the experimental fields every two weeks (8 -10 sampling campaigns),
* determination of soil pH in H_20
* determination of Hg content in soil bound with the following fractions:
  - water soluble,
  - exchangeable,
  - humic/fulvic.

Results and discussion

Research on the determination of the time of binding and mercury compounds stabilization in soil using various chemical substances was carried out. After every 2 weeks the sequential extraction was carried out to determine mercury fractions content in soil and soil pH in water. The results are presented in Tables 14-17.

Effects of pH

Changes in soil pH in H_2O versus type, quantity and time of the added chemical substances are shown in Table 14.

Table 14. Soil pH in H_2O

Sample	Start	2 week	4 week	6 week	8 week	10 week	12 week	14 week	16 week	18 week	20 week
soil - blank	7.81	7.72	7.73	7.71	7.71	7.74	7.71	7.77	7.72	7.70	7.67
soil + 0.5% fine sulfur	7.78	7.45	6.69	4.98	4.47	4.23	4.13	4.01	3.92	3.87	3.85
soil + 1% fine sulfur	7.77	7.33	6.32	4.58	4.31	4.11	3.99	3.88	3.81	3.73	3.69
soil +5% fine sulfur	7,52	6.83	5..96	4.1	4.09	3.92	3.85	3.77	3.68	3.61	3.59
soil + 1% granular sulfur	7.78	7.53	7.34	6.97	5.88	5.43	5,29	5.21	5.15	5.12	5.11
soil + 5% granular sulfur	7.71	7.38	6.85	5.67	5.34	4.91	4.76	4.64	4.52	4.45	4.42
soil + 10% granular sulfur	7.62	6.95	6.45	5.22	4.87	4.45	4.24	4.11	4.07	4.06	4.02
soil + 1% zeolite	7.79	7.65	7.66	7.75	7.65	7.72	7.75	7.71	7.69	7.63	7.65
soil + 5% zeolite	7.80	7.63	7.67	7.78	7.75	7.65	7.72	7.76	7.73	7.64	7.59
soil + 10% zeolite	7.78	7.66	7.74	7.91	7.98	7.95	7.91	7.78	7,81	7.73	7.61
soil + 1% mixture of dolomite and zeolite with brown coal	7.83	7.60	7.69	7.77	7.94	8.03	8.08	8.12	8.17	8.18	8.19
soil + 5% mixture of dolomite and zeolite with brown coal	7.87	7.61	7.73	7.93	8.21	8.43	8.51	8.56	8.61	8.68	8.70
soil + 10% mixture of dolomite and zeolite with brown coal	7.87	7.67	7.73	7.93	8.59	8.73	8.81	8.89	8.94	8.96	8.97

After 20 weeks soil pH in H_2O is at the same level for different quantities of zeolite in comparison to the control sample.

Maximum pH reduction in comparison to the control sample was noted in the case of adding 5% fine sulfur (about 4.2 pH unit) and 10% granular sulfur (about 3.8 pH unit). Figure 1 also indicates the effect of time on the changes in soil pH in H_2O as dependent on the type and quantity of sulfur. Similar changes in soil pH in H_2O for all of the added granulation of sulfur was identified. After the addition of different quantities and granulation of sulfur between the start and week 20 of the experiment three kinds of changes were observed. Between the start and 6 weeks of the experiment duration (for 1% granular sulfur between start and 8 week) a slow decrease in soil pH was observed. This is due to the reduction of unbound carbonates which regulate soil acidity. Soil pH value in KCl is 6.9 while the soil pH value in H_2O is 7.7 (Table 12). Six weeks into the experiment (for 1% granular sulfur- 8 weeks) a rapid decrease in soil pH was observed . which probably resulted from the change of buffer capacity. The third change, a slow decrease in soil pH in H_2O occurred between weeks 8 and 20 of the experiment. This phenomena probably occurs due to changes in the content of permanently bound structures in the soil structure.

Changes of soil pH in relation to the duration of the experiment, quantity of the added mixture of dolomite and zeolite with brown coal are illustrated in Figure 8. Between the start of the experiment and week 8 similar changes in soil pH were observed. This is probably an effect of the interaction between the chemical compounds contained in dolomite and the soil. For 8 weeks slight changes in the pH have been observed: a systematic increase in pH for the mixture of dolomite and zeolite with brown coal.

Stabilization/binding mercury compounds in soil

Results after the addition of various amendments to the mercury-contaminated soil are presented in tables 15 -17.

20 weeks after addition of various amendments to the mercury-contaminated soil, the content of mercury fraction that is water soluble was reduced (Table15). It was found that the type and quantity of the added chemical substances had no significant effect on the stabilization or binding of water soluble mercury compounds. Figures 9 and 10 show the effect of stabilization/binding of water soluble mercury fraction dependence on the type and quantity of various chemical substances. Similar changes in stabilization/binding of mercury fraction water soluble for all added substances were observed. Maximum reduction in comparison to the control sample was noted for all binding substances after 2 weeks. Two weeks of binding chemical application resulted in 43-78% reduction of water soluble mercury concentration in comparison to the control sample. Six weeks

of binding chemical application resulted in 72-95% reduction of water soluble mercury concentration in comparison to the control sample. Between 2 and 10 weeks of the experiment duration a slow decrease of water soluble mercury concentration was observed. For 12 weeks we have been observing slight changes in water soluble mercury concentrations.

The same phenomena were observed for nearly all added substances in the case of compounds bound exchangeable forms (Table 16 and Figures 11-12). With the addition of fine sulfur between weeks 12 and 20 of the experiment duration an increase of exchangeable mercury compounds content followed by its decrease were observed. It was found that the type and quantity of the added chemical substances had no significant effect on the stabilization or binding of exchangeable mercury compounds. Twenty weeks of binding chemical application resulted in 38-89% reduction of exchangeable mercury concentration in comparison to the control sample.

In the case of mercury compounds bound with fulvic and humic acid, no statistically significant differences after the addition of various stabilization/binding substances were observed in similar time (Table 17).

Summary

It was stated that in the case of 1% granular sulfur addition soil pH was changing at the slowest rate. This justifies the performance of an experiment with an addition of reduced granular sulfur and zeolite content: 0.5% granular sulfur and 0.5% zeolite into the soil. During the experiment the following were analyzed:

* pH soil in water extract,
* water soluble mercury compounds,
* exchangeable mercury compounds.

Table 19. Soil pH in water extract and contents of mercury compounds water soluble and exchangeable (mg/kg; n = 3).

Sample	pH				water soluble				exchangeable			
	Start	2 week	4 week	6 week	Start	2 week	4 week	6 week	Start	2 week	4 week	6 week
soil - blank	7.57	7.53	7.51	7.53	0.486 ± 0.078	0.510 ± 0.073	0.493 ± 0.075	0.521 ± 0.069	3.346 ± 0.62	3.208 ± 0.60	3.296 ± 0.61	3.311 ± 0.59
soil + 0.5% granular sulfur	7.53	7.51	7.49	7.45	0.503 ± 0.081	0.273 ± 0.054	0.185 ± 0.038	0.110 ± 0.022	3.419 ± 0.65	3.306 ± 0.61	3.118 ± 0.62	3.232 ± 0.61
soil + 0.5% zeolite	7.51	7.54	7.52	7.51	0.478 ± 0.074	0.315 ± 0.051	0.275 ± 0.055	0.247 ± 0.048	3.277 ± 0.59	3.419 ± 0.64	3.323 ± 0.63	3.152 ± 0.60

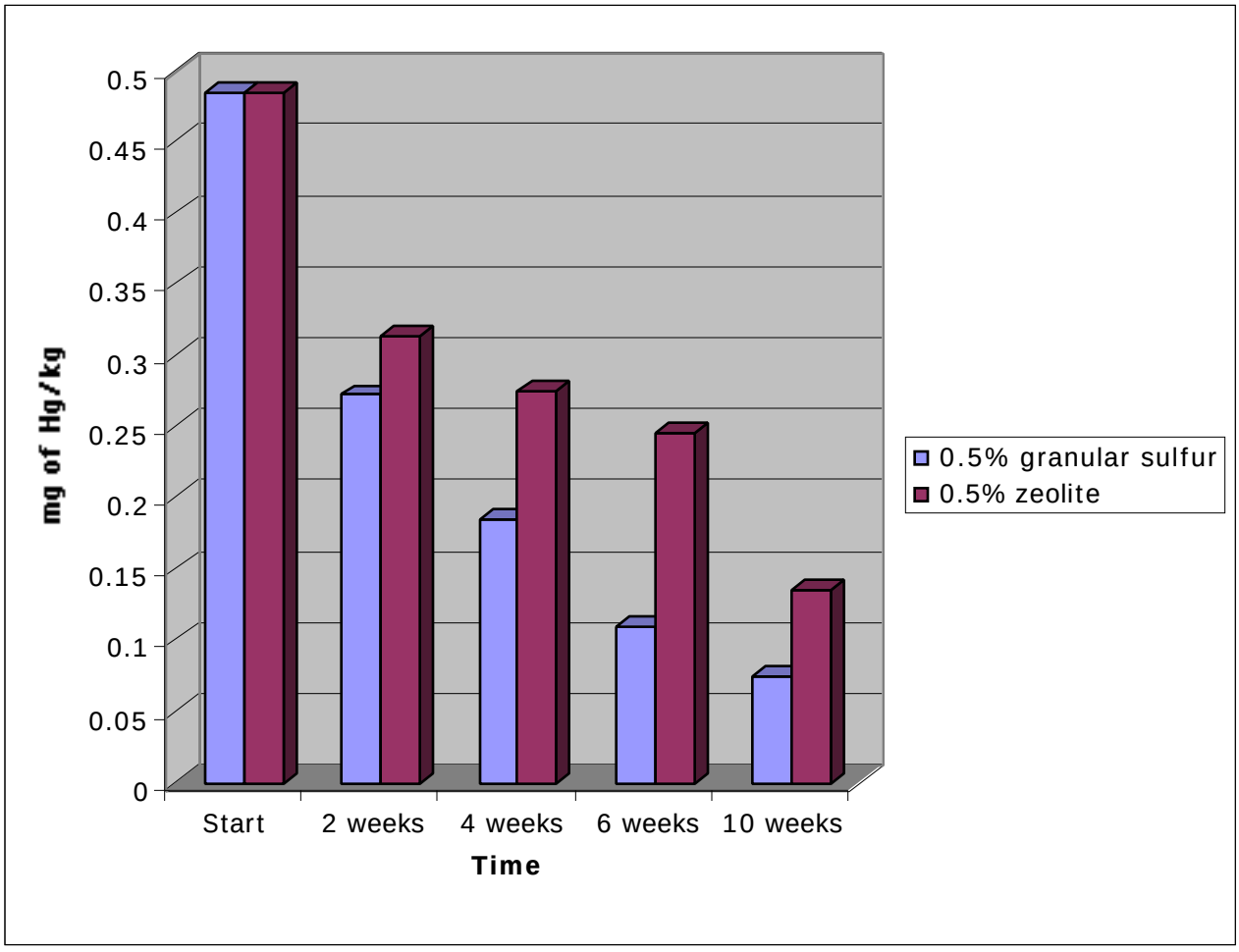


Fig. 13. Changes of mercury water soluble fraction in soil depending on binding substance

In the case of the addition 0.5% granular sulfur, between the start and 6 weeks of the experiment duration a slight decrease of soil was observed. Six weeks of binding chemical application resulted in 49% for 0.5% zeolite to 78% for 0.5% granular sulfur reduction of water soluble mercury concentration in comparison to the control sample.

Table 15. Content of mercury water soluble fraction in soil [mg/kg soil]

Sample	Start	2 week	4 week	6 week	8 week	10 week	12 week	14 we
soil - blank	0.447 ± 0.081	0.409 ± 0.078	0.421 ± 0.080	0.410 ± 0.079	0.431 ± 0.082	0.422 ± 0.079	0.442 ± 0.075	0.433 0.07
soil + 0.5% fine sulfur	0.393 ± 0.077	0.152 ± 0.029	0.124 ± 0.025	0.066 ± 0.014	0.049 ± 0.011	0.038 ± 0.009	0.029 ± 0.007	0.023 0.00
soil +1% fine sulfur	0.369 ± 0.074	0.210 ± 0.038	0.179 ± 0.038	0.118 ± 0.024	0.096 ± 0.018	0.078 ± 0.016	0.052 ± 0.012	0.033 0.00
soil + 5% fine sulfur	0.360 ± 0.078	0.255 ± 0.042	0.199 ± 0.034	0.103 ± 0.021	0.073 ± 0.015	0.054 ± 0.013	0.038 ± 0.011	0.023 0.00
soil + 1% granular sulfur	0.411 ± 0.079	0.179 ± 0.035	0.154 ± 0.042	0.136 ± 0.026	0.088 ± 0.017	0.069 ± 0.015	0.044 ± 0.009	0.034 0.00
soil + 5% granular sulfur	0.398 ± 0.072	0.155 ± 0.039	0.098 ± 0.022	0.110 ± 0.024	0.079 ± 0.018	0.063 ± 0.015	0.042 ± 0.009	0.029 0.00
soil + 10% granular sulfur	0.376 ± 0.075	0.159 ± 0.040	0.108 ± 0.023	0.085 ± 0.017	0.076 ± 0.017	0.072 ± 0.016	0.046 ± 0.010	0.035 0.00
soil + 1% zeolite	0.358 ± 0.071	0.160 ± 0.041	0.115 ± 0.021	0.107 ± 0.019	0.091 ± 0.020	0.083 ± 0.019	0.068 ± 0.015	0.047 0.01
soil + 5% zeolite	0.368 ± 0.073	0.127 ± 0.025	0.131± 0.025	0.125 ± 0.023	0.108 ± 0.021	0.093 ± 0.020	0.071 ± 0.015	0.045 0.00
soil + 10% zeolite	0.377 ± 0.072	0.128 ± 0.025	0142 ± 0.028	0.113 ± 0.022	0.097 ± 0.019	0.085 ± 0.017	0.074 ± 0.013	0.048 0.00

Sample	Start	2 week	4 week	6 week	8 week	10 week	12 week	14 we
soil + 1% mixture of dolomite and zeolite with brown coal	0.385 ± 0.075	0.130 ± 0.026	0.121 ± 0.023	0.098 ± 0.019	0.086 ± 0.017	0.082 ± 0.017	0.069 ± 0.014	0.054 0.00
soil + 5% mixture of dolomite and zeolite with brown coal	0.382 ± 0.076	0.133 ± 0.024	0.127 ± 0.023	0.108 ± 0.020	0.091 ± 0.018	0.083 ± 0.017	0.074 ± 0.012	0.072 0.01
soil + 10% mixture of dolomite and zeolite with brown coal	0.372 ± 0.077	0.128 ± 0.026	0.118 ± 0.019	0.115 ± 0.021	0.102 ± 0.020	0.096 ± 0.017	0.085 ± 0.016	0.079 0.01

Table 16. Content of mercury exchangeable fraction in soil [mg/kg soil]

Sample	2 week	4 week	6 week	8 week	10 week	12 week	14 wee
soil - blank	2.988 ± 0.59	3.015 ± 0.62	3.126 ± 0.63	3.096 ± 0.64	3.211 ± 0.66	3.087 ± 0.61	3.303 ± 0
soil + 0.5% fine sulfur	2.736 ± 0.61	3.796 ± 0.75	1.568 ± 0.21	1.783 ± 0.28	1.635 ± 0.25	1.341 ± 0.21	1.270 ± 0
soil +1% fine sulfur	2.938 ± 0.64	5.718 ± 0.98	1.726 ± 0.25	1.684 ± 0.26	1.778 ± 0.27	1.471 ± 0.23	1.288 ± 0
soil + 5% fine sulfur	3.034 ± 0.57	9.486 ± 1.35	2.015 ± 0.37	1.915 ± 0.34	1.843 ± 0.31	1.653 ± 0.25	1.562 ± 0
soil + 1% granular sulfur	3.049 ± 0.60	4.023 ± 0.84	1.856 ± 0.29	1.807 ± 0.31	1.802 ± 0.32	2.045 ± 0.28	2.169 ± 0
soil + 5% granular sulfur	2.954 ± 0.58	3.512 ± 0.69	1.421 ± 0.22	1.361 ± 0.25	1.268 ± 0.24	1.183 ± 0.22	1.143 ± 0
soil + 10% granular sulfur	2.873 ± 0.59	3.018 ± 0.64	1.395 ± 0.23	1.299 ± 0.23	1.367 ± 0.26	1.309 ± 0.21	1.167 ± 0
soil + 1% zeolite	2.986 ± 0.61	3.457 ± 0.68	1.153 ± 0.21	1.066 ± 0.22	1.108 ± 0.23	1.219 ± 0.20	1.268 ± 0
soil + 5% zeolite	3.156 ± 0.64	3.681 ± 0.73	1.130 ± 0.22	1.109 ± 0.21	1.056 ± 0.22	1.196 ± 0.19	1.230 ± 0
soil + 10% zeolite	2.684 ± 0.59	3.798 ± 0.76	1.117 ± 0.23	1.078 ± 0.22	1.095 ± 0.23	1.189 ± 0.18	1.256 ± 0
soil + 1% mixture of dolomite and zeolite with brown coal	2.914 ± 0.55	3.731 ± 0.77	1.191 ± 0.21	1.272 ± 0.25	1.220 ± 0.25	1.109 ± 0.16	1.194 ± 0
soil +5% mixture of dolomite and zeolite with brown coal	2.825 ± 0.57	4.139 ± 0.81	1.220 ± 0.20	1.305 ± 0.25	1.394 ± 0.27	1.229 ± 0.19	1.264 ± 0
soil + 10% mixture of dolomite and zeolite with brown coal	2.707 ± 0.61	3.986 ± 0.79	1.288 ± 0.22	1.433 ± 0.27	1.133 ± 0.21	1.213 ± 0.19	1.327 ± 0

Table 17. Content of mercury humic/fulvic fraction [mg/kg soil] (n=

Sample	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	14 wee
soil - blank	31.36 ± 4.22	33.08 ± 4.12	32.44 ± 4.09	34.28 ± 4.02	35.16 ± 3.68	33.28 ± 2.87	31.96 ± 2
soil + 0.5% fine sulfur	36.42 ± 4.29	35.15 ± 4.26	37.26 ± 3.98	36.08 ± 4.15	38.15 ± 3.54	36.21 ± 3.61	34.13 ± 3
soil +1% fine sulfur	33.08 ± 4.15	34.19 ± 4.19	32.36 ± 4.01	35.16 ± 4.12	33.95 ± 4.07	34.27 ± 2.99	32.45 ± 3
soil + 5% fine sulfur	32.15 ± 4.08	31.28 ± 4.23	34.08 ± 4.13	33.45 ± 4.11	32.78 ± 4.15	33.89 ± 3.17	35.23 ± 3
soil + 1% granular sulfur	29.86 ± 3.72	31.38 ± 4.02	32.18 ± 4.07	30.63 ± 4.22	32.38 ± 4.18	31.73 ± 2.89	34.37 ± 3
soil + 5% granular sulfur	31.43 ± 4.06	30.56 ± 3.81	31.74 ± 3.92	32.44 ± 4.26	33.06 ± 4.27	31.88 ± 2.91	33.15 ± 2
soil + 10% granular sulfur	33.72 ± 4.17	34.88 ± 4.02	32.42 ± 3.95	35.02 ± 4.31	33.18 ± 4.24	33.34 ± 3.14	35.58 ± 3
soil + 1% zeolite	35.61 ± 4.27	34.16 ± 3.97	36.72 ± 4.11	35.21 ± 4.18	37.02 ± 4.34	35.08 ± 3.24	38.03 ± 3
soil + 5% zeolite	32.43 ± 4.15	33.95 ± 3.91	32.18 ± 4.06	34.24 ± 4.01	34.66 ± 4.05	33.94 ± 3.84	35.49 ± 3
soil + 10% zeolite	33.65 ± 4.21	32.19 ± 4.03	34.36 ± 3.89	33.36 ± 4.12	32.13 ± 4.16	31.41 ± 3.18	34.06 ± 3
soil + 1% mixture of dolomite and zeolite with brown coal	30.72 ± 4.16	32.76 ± 3.88	31.09 ± 4.06	33.14 ± 3.93	31.63 ± 4.03	30.96 ± 3.34	33.72 ± 3
soil + 5% mixture of dolomite and zeolite with brown coal	32.46 ± 4.22	34.51 ± 3.74	35.35 ± 3.69	34.66 ± 3.78	35.06 ± 3.69	37.18 ± 3.66	35.98 ± 3
soil + 10% mixture of dolomite and zeolite with brown coal	29.78 ± 3.96	30.98 ± 4.01	32.37 ± 4.11	33.78 ± 4.09	31.96 ± 4.12	33.53 ± 3.78	34.47 ± 3

Fig. 2 Changes mercury emission values in the period 1991 .

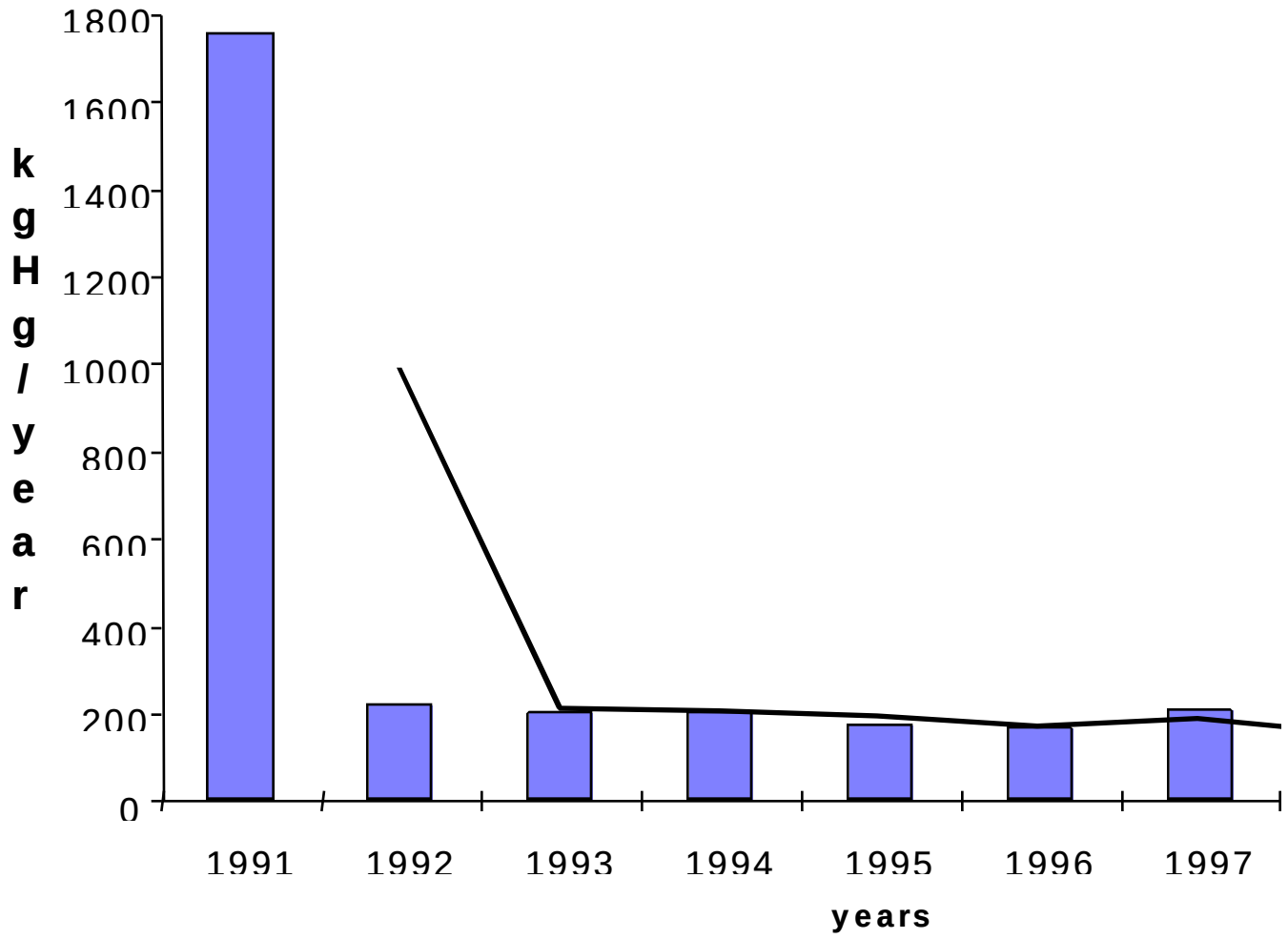


Fig. 3 Mercury deposition

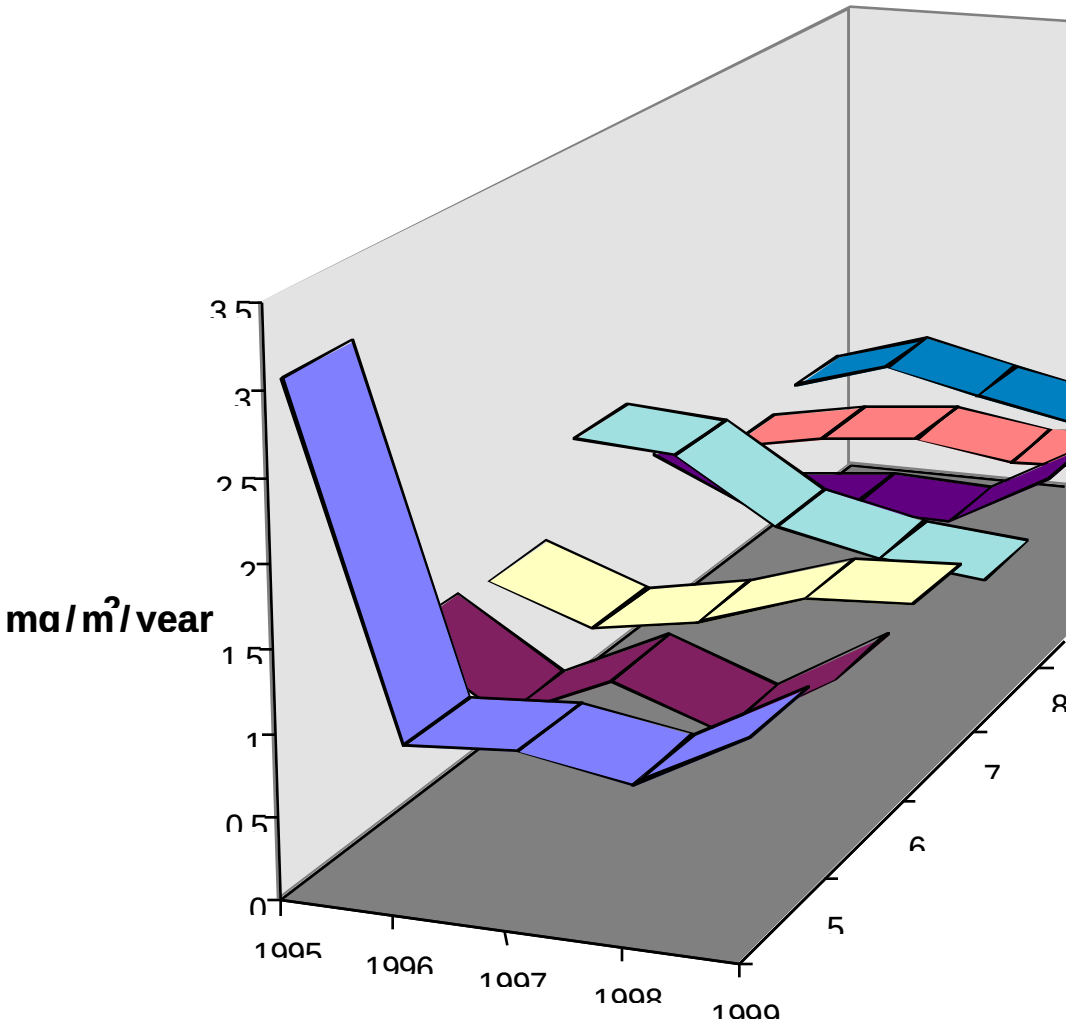


Fig. 4 Mercury concentration in Dunaiec River

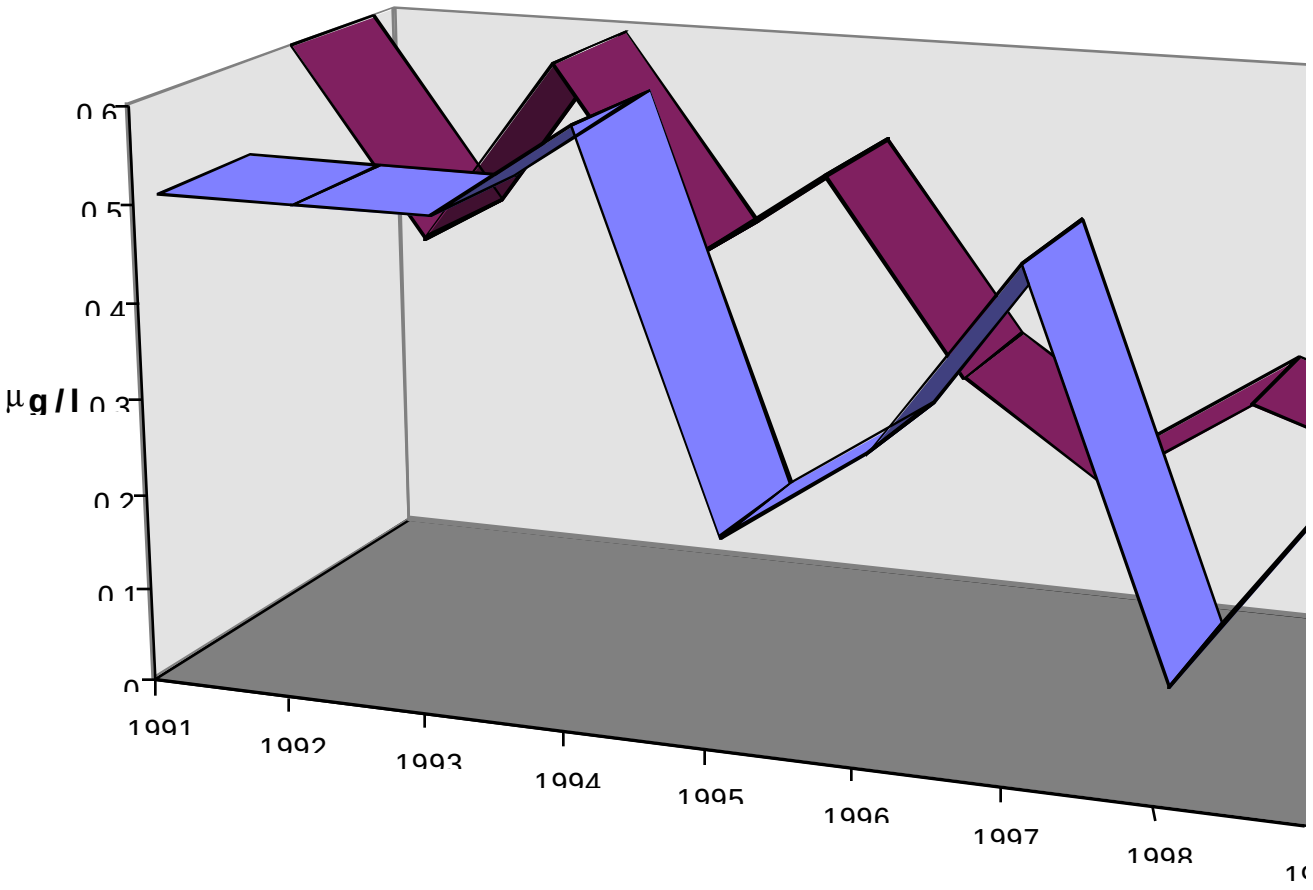
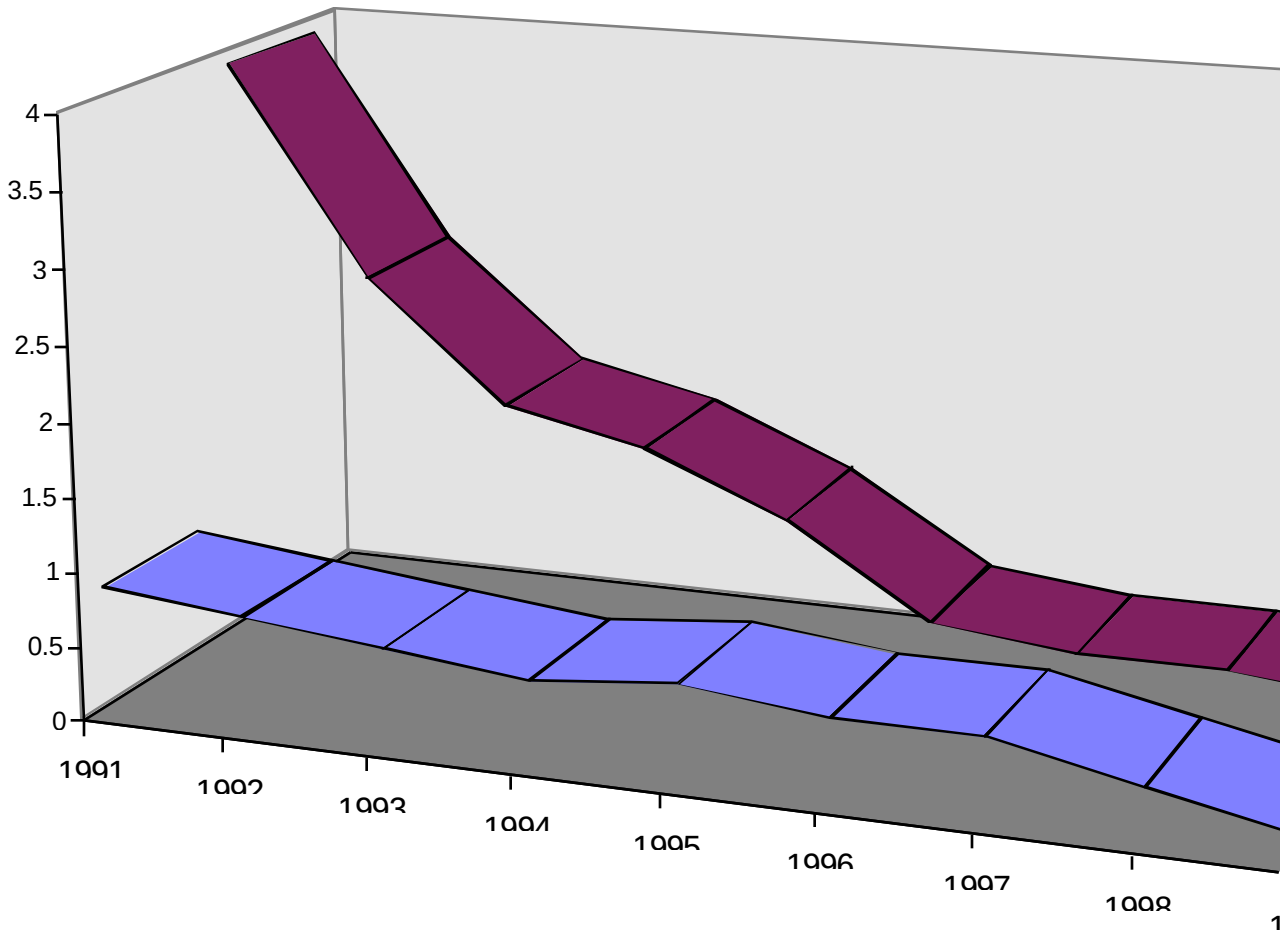


Fig. 5 Mercury concentration in Biala Ryer



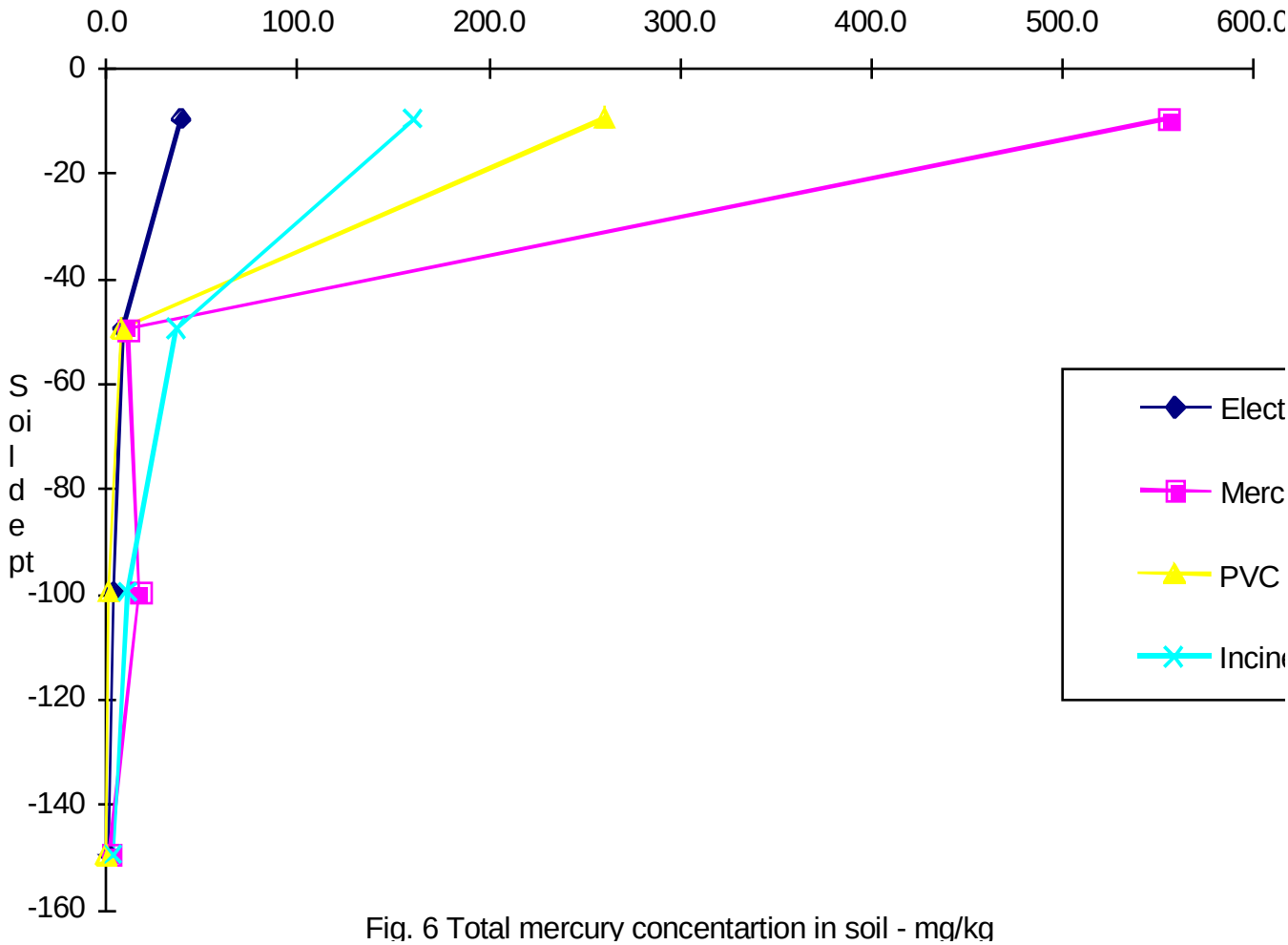


Fig. 6 Total mercury concentartion in soil - mg/kg

Fig. 7 Changes in soil pH/H_2O vs granulation of sulfur

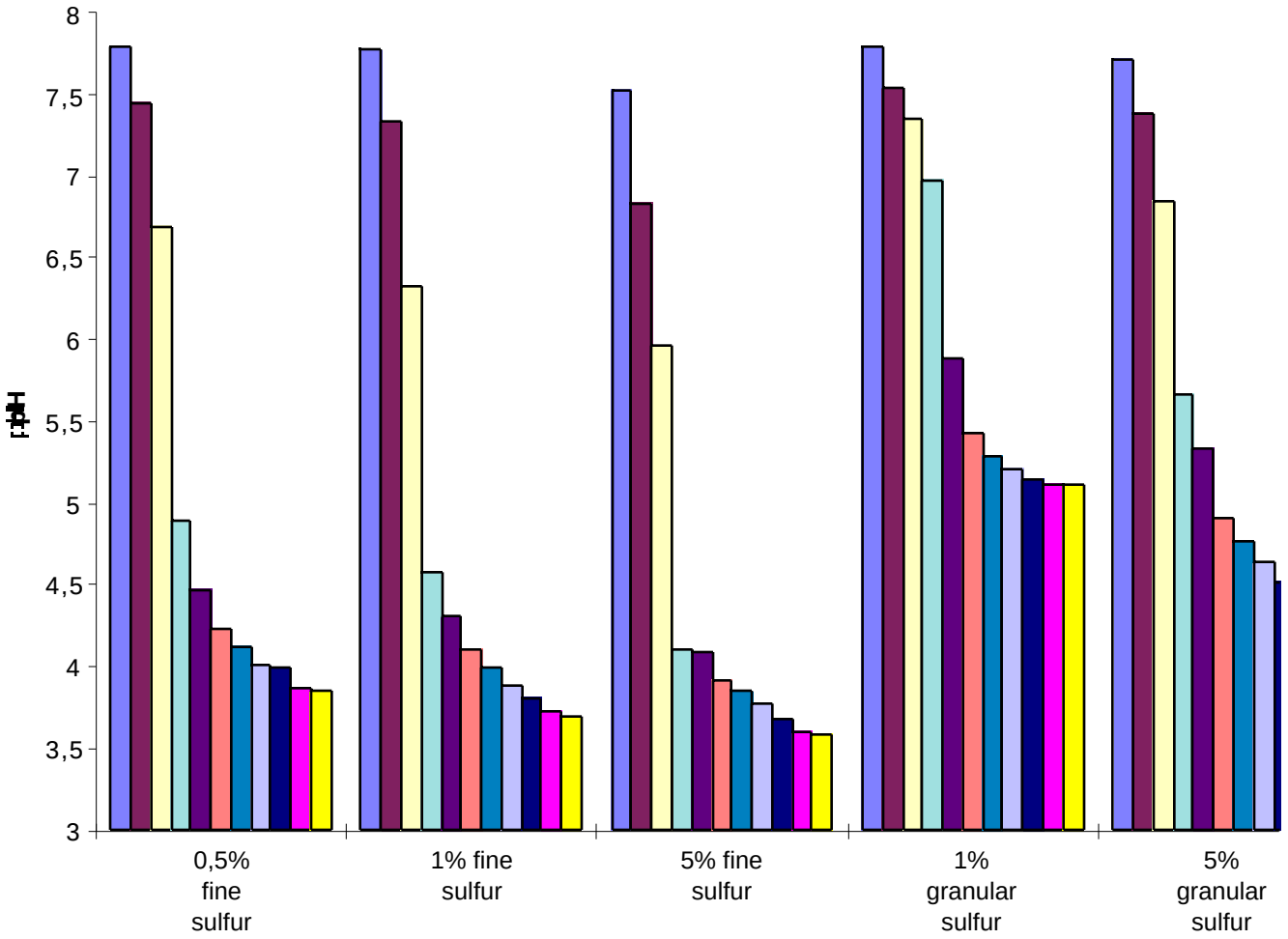


Fig. 8 Changes in soil pH/H_20 vs quantity of mixture dolomite and zeolite w

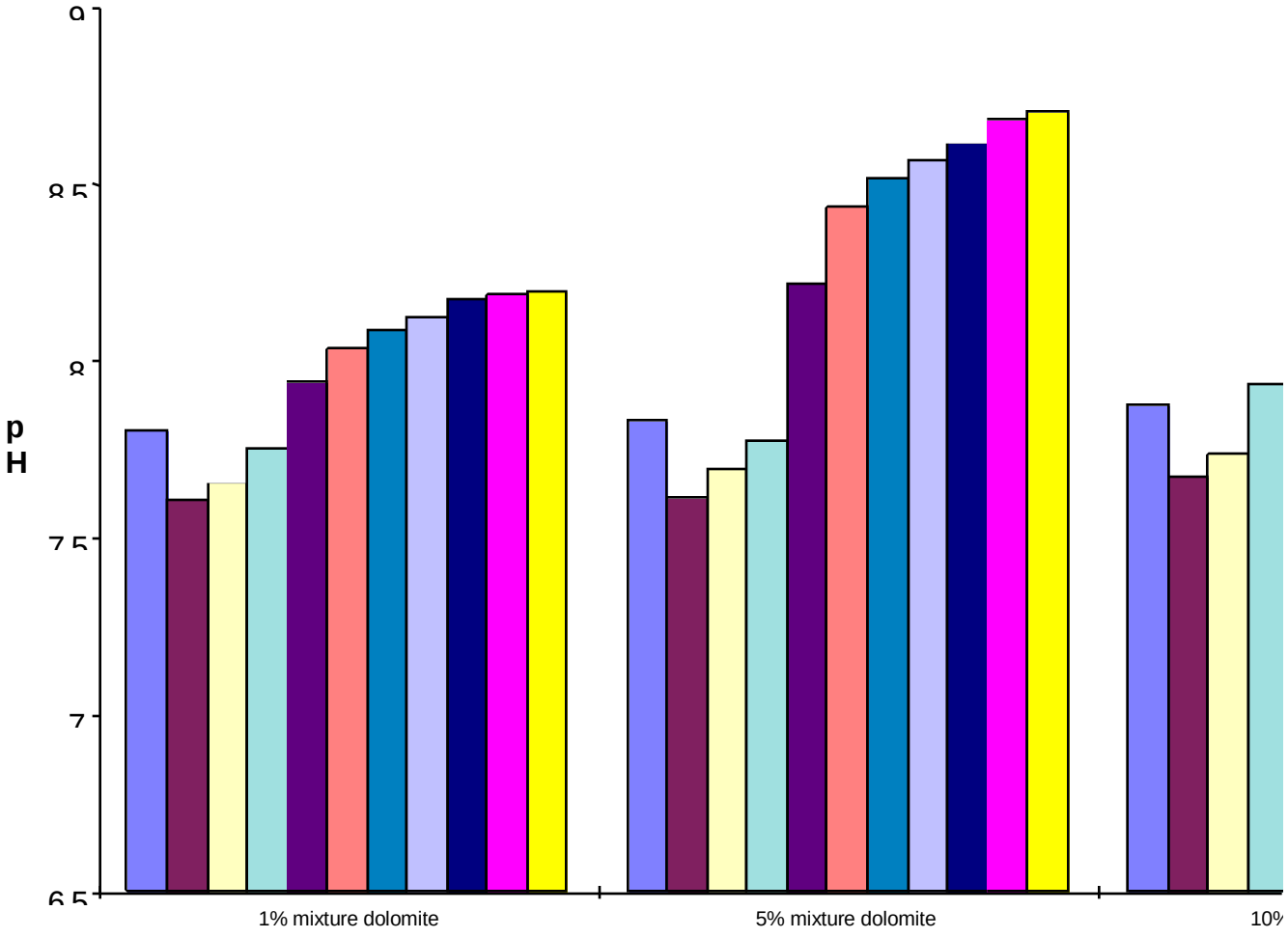


Fig. 9 Changes of mercury water soluble fraction in soil deppending on grai

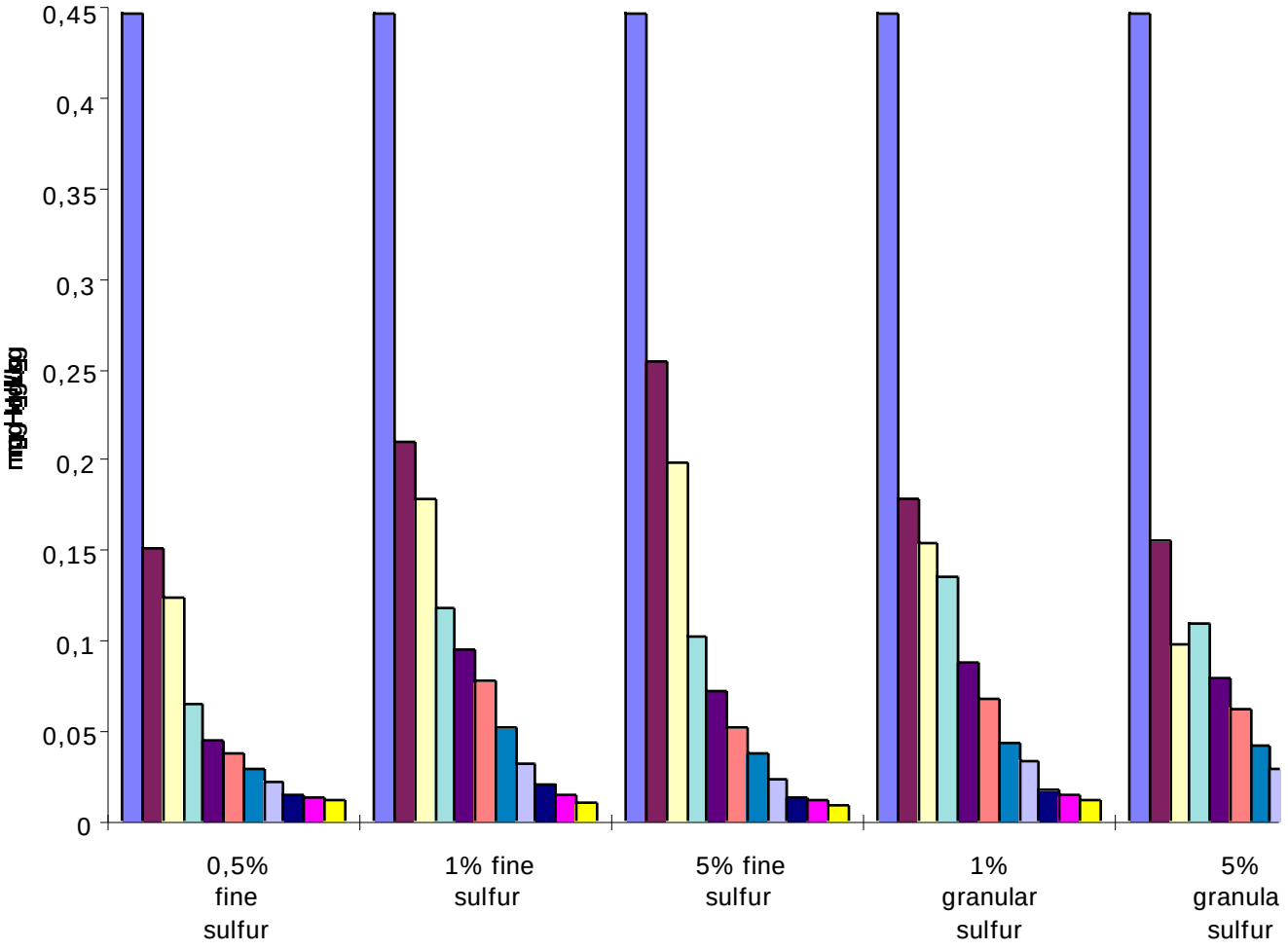


Fig. 10 Changes of mercury water soluble fraction in soil depending on zeolite a and zeolite with brown carbon

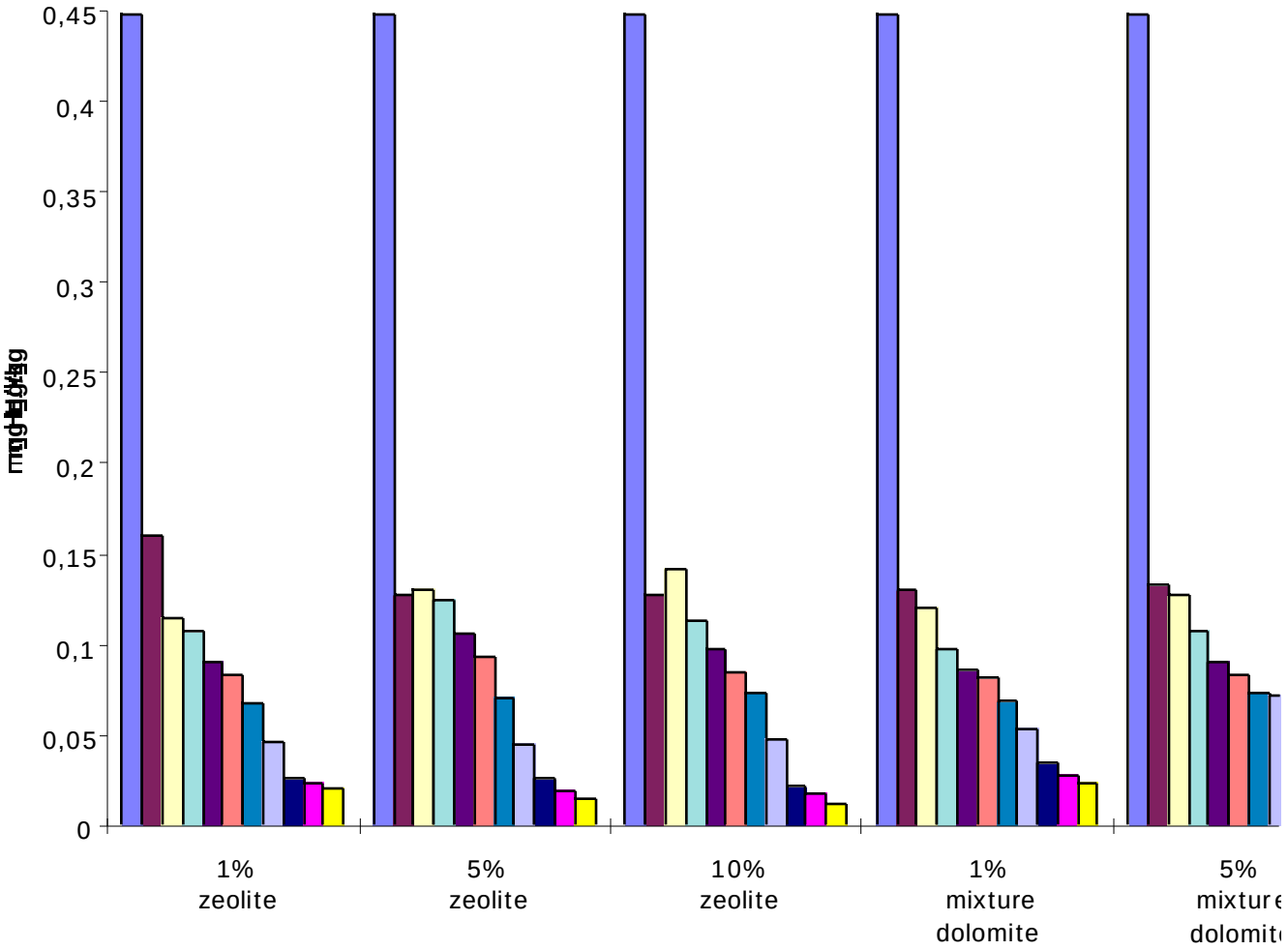


Fig. 11 Changes of mercury exchangeable fraction in soil depending on gra

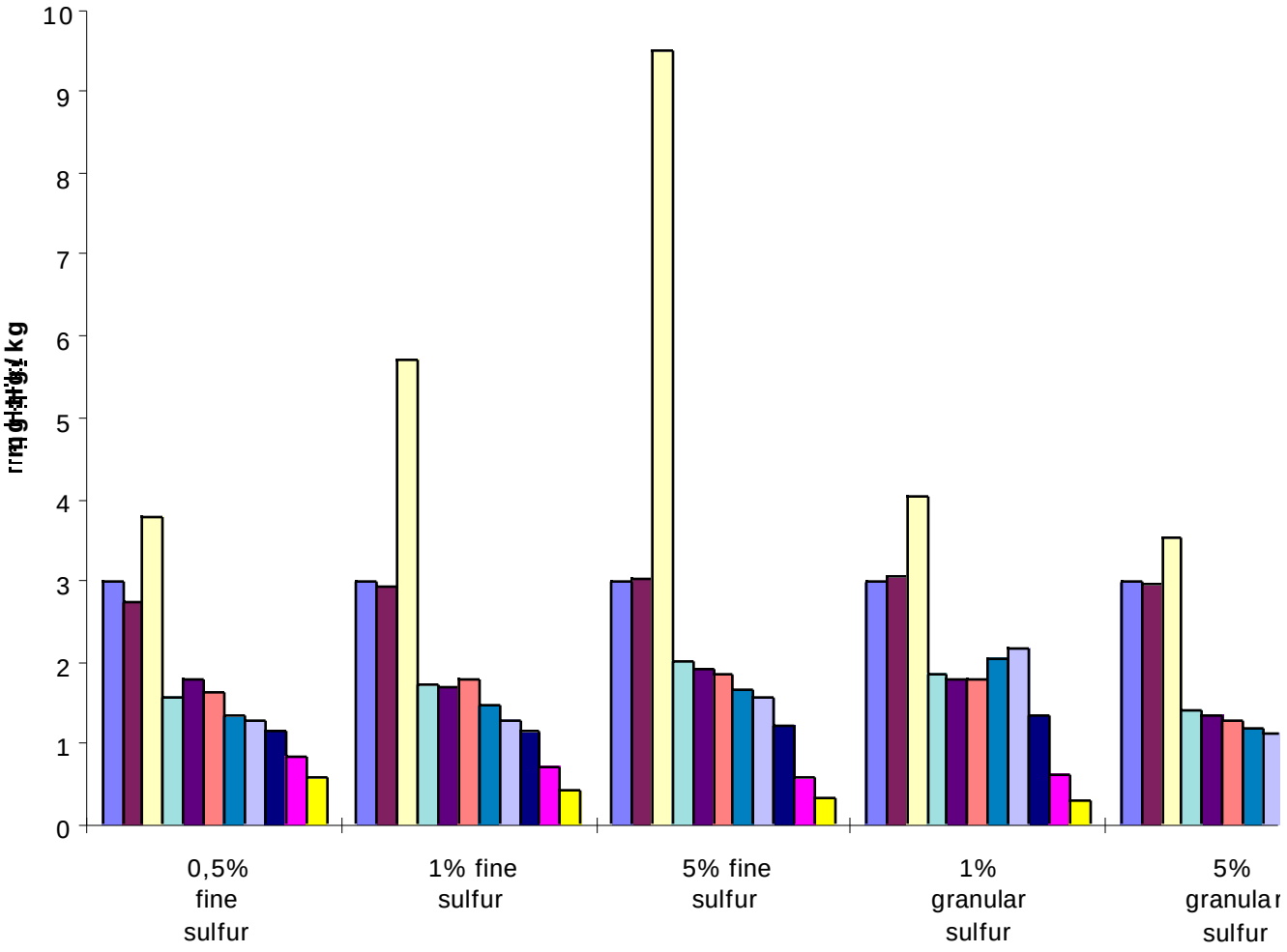
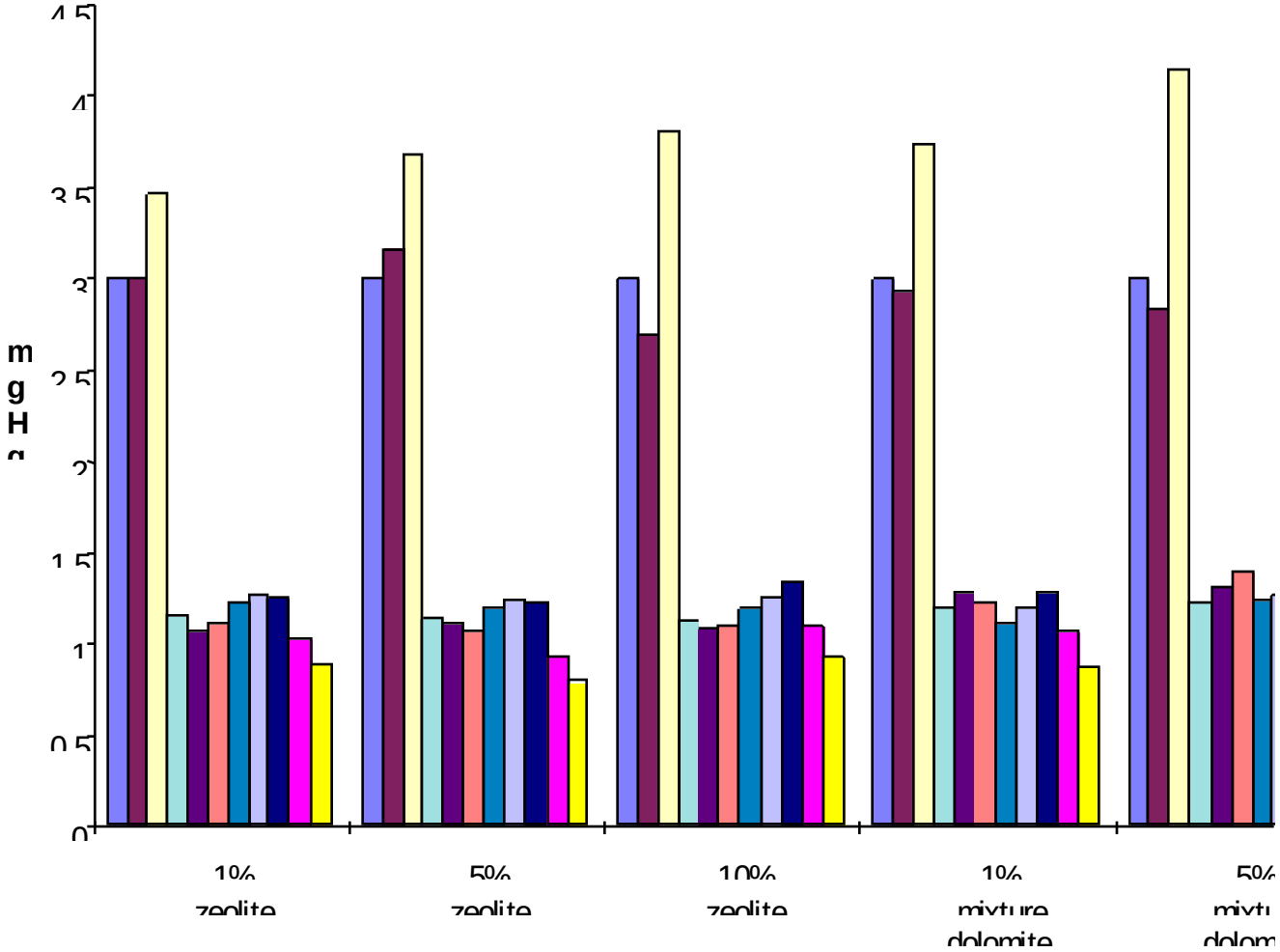


Fig. 12 Changes of mercury exchangeable in soil depeding on zelite and m
zeolite with brown coal



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Project Deliverable

Phytoremediation Project

FY00 Final Report – Appendix 2

Evaluation of Novel Mercury Remediation Technology

Submitted to:
U.S. Department of Energy
Federal Energy Technology Center
Morgantown, West Virginia

Submitted by:
Institute for Ecology of Industrial Areas
Katowice, Poland

Institute for Central and Eastern European Cooperative Environmental Research
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Mercury resistant plant species screening of soil microbial/fungal activity and stabilization using plants and microbes

Living plants have the ability to accumulate heavy metals, in particular those metals which are essential for growth and development, from soil water solution. Certain plants also have the ability to accumulate heavy metals, which have no known biological function (e.g., Cd, Hg). However, excessive accumulation of these metals can be toxic to most plants. Heavy metal ions, when present at an elevated level in the environment, are adsorbed by roots and translocated to different plant parts, leading to impaired metabolism and reduced growth.

The roots of plants interact with a large number of different microorganisms. Microbial populations are invariably higher in the rhizosphere than in root-free soil. Populations of bacteria in field soils may exceed 100 million g^{-1} as estimated by soil dilution and plate counts. From such counts the rhizosphere/non-rhizosphere ratio (R/S) values are most frequently in the range of 2–20. The ratio of fungi to bacteria biomass in an arable soil was found to be 5–10, and the ratio of percent root surface covered by fungi/percent covered by bacteria has been estimated to range from 0.28–14.0. Most prevalent in the rhizosphere are *Pseudomonas spp.* of which *Pseudomonas. fluorescens* and *P. putida*) are known to be able to enhance plant growth.

Soil actinomycetes, predominantly *Streptomycess* spp., are usually second to bacteria in populations, with common R/S ratios of 5 to 10. Actinomycetes are best known for antibiotic production and the inhibition of plant pathogens and other soil microorganisms.

In general, populations of fungi are lower than those of bacteria and actinomycetes. The R/S ratios derived from plate counts may range from 3:1 to > 100:1 but most frequently are 10:1 to 20:1 for the rhizosphere crop plants.

Mercury is considered a toxic pollutant unnecessary for plant biological functions. Mercuric ions (Hg^{2+}) form methylated derivatives, which are stable in aqueous solution. Methylated derivatives of Hg^{2+} and inorganic Hg^{2+} have a high affinity for –SH and –S-S groups, which are ubiquitous in living organisms (e.g., in enzymes and other proteins). Therefore, the toxic effects of Hg^{2+} are the result of the ability of Hg^{2+} to bind to these important functional groups of biological molecules. Methylated forms of Hg^{2+} also have been shown also to bind to the nitrogen of nucleotide bases in RNA and DNA, where they could interfere with the functioning of these nucleic acids.

Bacterial cells have coexisted with toxic heavy metals, (e.g. Hg), since the origin of life. They have mechanisms of resistance to common toxic heavy metals (including Hg). Both Gram positive and Gram negative bacteria have systems for detoxifying inorganic Hg^{2+}.

Some bacteria can convert Hg^{2+} (the highly reactive and toxic water-soluble ionic form) into Hg^{o}(the elemental form). The elemental form of mercury is much less toxic than the mercurous ion, and the physical properties of Hg^{o} greatly decrease its availability to microbes. Hg^{o} is virtually insoluble in water and has a high vapor pressure, which leads to rapid evaporation. This microbial mechanism is induced at picomolar Hg concentrations found in natural systems (water/soil).

The generally accepted mechanism of bacterial resistance to mercuric ions involves intracellular reduction of Hg^{2+} to Hg^{o} by mercuric reductase, with subsequent volatilization. Mercuric reductases have been isolated from a number of microorganisms, including *Escherichia coli, Thiobacillus ferrooxidans, Streptomyces, Streptococcus,* and *Caulobacter*.

Some microorganisms might also be able to oxidize Hg^{o} to Hg^{2+}, e.g., two typical soil bacteria *Bacillus* and *Streptomyces* oxidize Hg^{o} to Hg^{2+}. Hg^{o} is also oxidized to Hg^{2+} in the atmosphere as a result of its interaction with ozone in the presence of water. It has also been known for over a decade that mammals and plants effectively oxidize monatomic Hg^{o} vapor to Hg^{2+} using catalase, and, possibly peroxidases.

Methyl mercury is a serious concern associated with mercury contamination. This concern is a result of the extreme neurotoxicity of methyl mercury to humans at very low concentrations. The production of methyl mercury has long been considered primarily a biological process, occurring within aquatic sediments via bacterial methylation of inorganic mercury. Many microorganisms display mercury-methylating activity in pure cultures, including *Clostridium*, *Neurospora*, *Pseudomonas, Bacillus, Mycobacterium, Escherichia coli, Aerobacter aerogenes, Bacillus megaterium,* and a number of fungi. Methylation occurs most rapidly in the presence of active microbial sulfate reduction. Production of sulfide is inhibited by methylation process above optimal sulfate concentration, whereas at lower sulfate levels microbial sulfate reduction and hence mercury methylation are limited by available sulfate.

Bacteria play an important role in mercury cycling leading to increased bacterial methylation of mercury and subsequent bioaccumulation in higher organisms. It is also well known that roots of plants create different microflora depending of plant species.

The goal of the task was to screen the microbial populations associated with the rhizosphere of different plant species for their reaction to mercury-contaminated soils (soil properties are presented in Table 1). We approached this goal by determining:

- the types of microorganisms,
- the reaction of the microbes to mercury-contaminated soil,
- the ratio of the number of microbes in the rhizosphere to the number in the root-free soil (R/S) for different plant species.

The plant experiments were performed in containers located in growth chambers, which are controlled for light and temperature.

Table 1. Soil properties

Investigated Component	**Unit**	**Mean ± SD**
PH_{H2O}		7.70 ± 0.00
pH_{KCl}		6.92 ± 0.02
Electrical Conductivity	µS/cm	201.5 ± 1.5
Cation Exchange Capacity	mol/kg	15.27 ± 1.36
Organic Carbon	%	1.77 ± 0.14
Organic Matter	%	3.05 ± 0.23
Total:		
N	%	0.105 ± 0.00
P_2O_5	mg $100g^{-1}$	0.137 ± 0.01
K_2O	mg $100g^{-1}$	12.2 ± 0.11

Soil moisture content regime was maintained at 60% of total water capacity. Because of the high content of soil phosphorus and potassium, only nitrogen fertilizer was applied. The species utilized in the experiment are presented below. The test plants were planted on Hg contaminated soil in four replications.

Fertilization	
Hg-contaminated soil planted with plants A, B, C, D, E	Hg–contaminated soil blank
plant A: *Helianthus tuberosus* plant B: *Armoracia lapathifolia* plant C: *Poa pratensis* plant D: *Festuca rubra* plant E: *Salix viminalis*	

The growth, yield, health and vitality of investigated plant species were reported, as well as mercury distribution in different parts of the plant.

Time of root rhizosphere development was established as one month. That means that plants were grown for one month in order to establish a rhizosphere prior to starting the experiments. The soil was sampled inside and outside of the rhizosphere for each pot. Samples were collected after one month of experiment (7 of July), after the first crop of *Festuca rubra* and *Poa pratensis (8 of August)* and in the end of experiment (18 of August for *Helianthus tuberosus,* 11 of August for *Armoracia lapathifolia*, 2 of October for *Poa pratensis* and *Festuca rubra*, 5 of October for *Salix viminalis*). A garden soil was used as a control medium for microbiological investigation. Samples were microbiologically examined in order to determine:

- total number of soil bacteria [10% tryptic soy agar (TSA, Difco)];
- number of *Pseudomonas* on the selective medium (Grant and Holt, 1977);
- number of soil fungi on Czapek-Dox medium with rose bengal (Alef, 1995);
- number of protein decomposing bacteria (by the MPN method);
- number of sulfur amino-acid decomposing bacteria;
- number of ammonificators; and
- number of nitrificators.

The results were expressed as colony-forming units per gram of dry soil (CFU g^{-1}). All soil samples are analyzed for soil pH (H_2O and KCl) using the **ISO 10390** method.

The soil samples from each container were divided into:

- root -free soil – 10g of soil were transferred to 250ml Erlenmeyer flasks containing 90mL 0.85% NaCl. Flasks were shaken on a rotary shaker for 10 min at 120 rev min^{-1}. The soil suspension was used for the determination of the root-free soil microbial population.
- rhizosphere soil - plants were removed from the soil and roots along with adhering soil were cut into small pieces for analysis. Samples of roots (5g) were transferred to 250ml Erlenmeyer flasks containing 45ml 0.85% NaCl. Flasks were shaken on a rotary shaker for 10 min at 120 rev min^{-1}. The soil suspension was used for the determination of the rhizosphere microbial population.

Microbial populations were determined in each soil sample. Root-free soil and rhizosphere samples were serially diluted in 0.85% NaCl and appropriate dilutions were plated on 10% tryptic soy agar (TSA), on the selective medium for *Pseudomonas*, on Czapek-Dox medium with rose bengal and on Frazier medium. The numbers of nitrifying bacteria, ammonifying bacteria and sulfur-amino acid-decomposing bacteria were estimated by the MPN method. One milliliter samples of the dilution series (up to 10^{-8}) were pipetted into tubes (3 replicate tubes per dilution) containing a specific medium. The tubes were carefully mixed (by hand rolling) and incubated. At the end of the incubation period, the tubes were observed for growth, for a specific color (after adding a specific reagent to ammonium, nitrite and nitrate) or for blackening (sulfur-amino acid-decomposing bacteria). The results are shown in Table 2 - 6.

Plant species screening

After preliminary investigations the following plant species were chosen for the experiment: *Festuca rubra* (fescue), *Poa pratensiss* (meadow grass), *Armoracia lapathifolia* (horseradish), *Salix viminalis* (willow), *Helianthus tuberosus* (sunflower also known as Jerusalem artichoke). Grasses were planted from seeds, horseradish from parts of roots, sunflower from bulbs and willow from seedlings. Seed germination of fescue and meadow grass, as well as plant growth, yield, health, vitality and mercury distribution in roots and shoots were investigated and analyzed for each plant. Results are presented in Figures 1, 2 and 3.

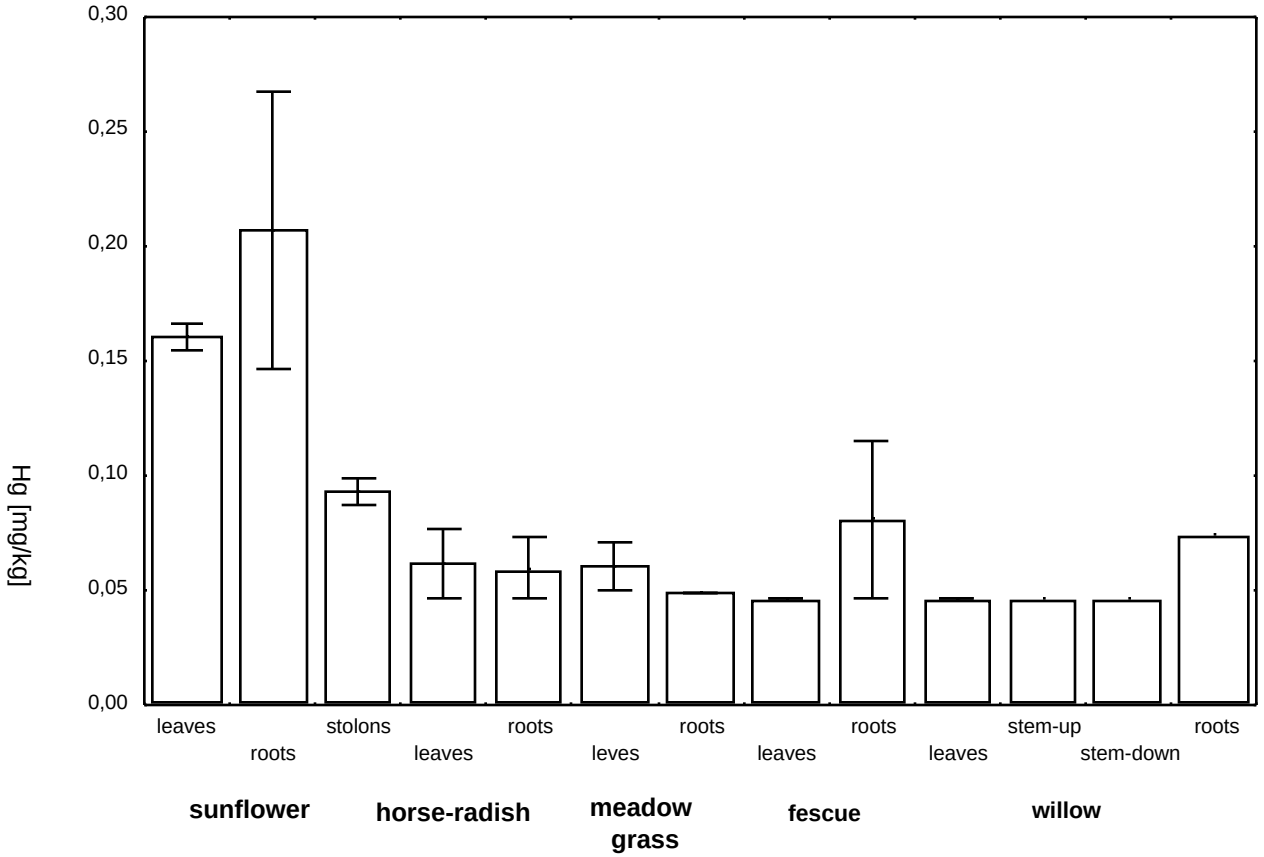

Figure 1. Concentration of mercury in plants on garden soil.

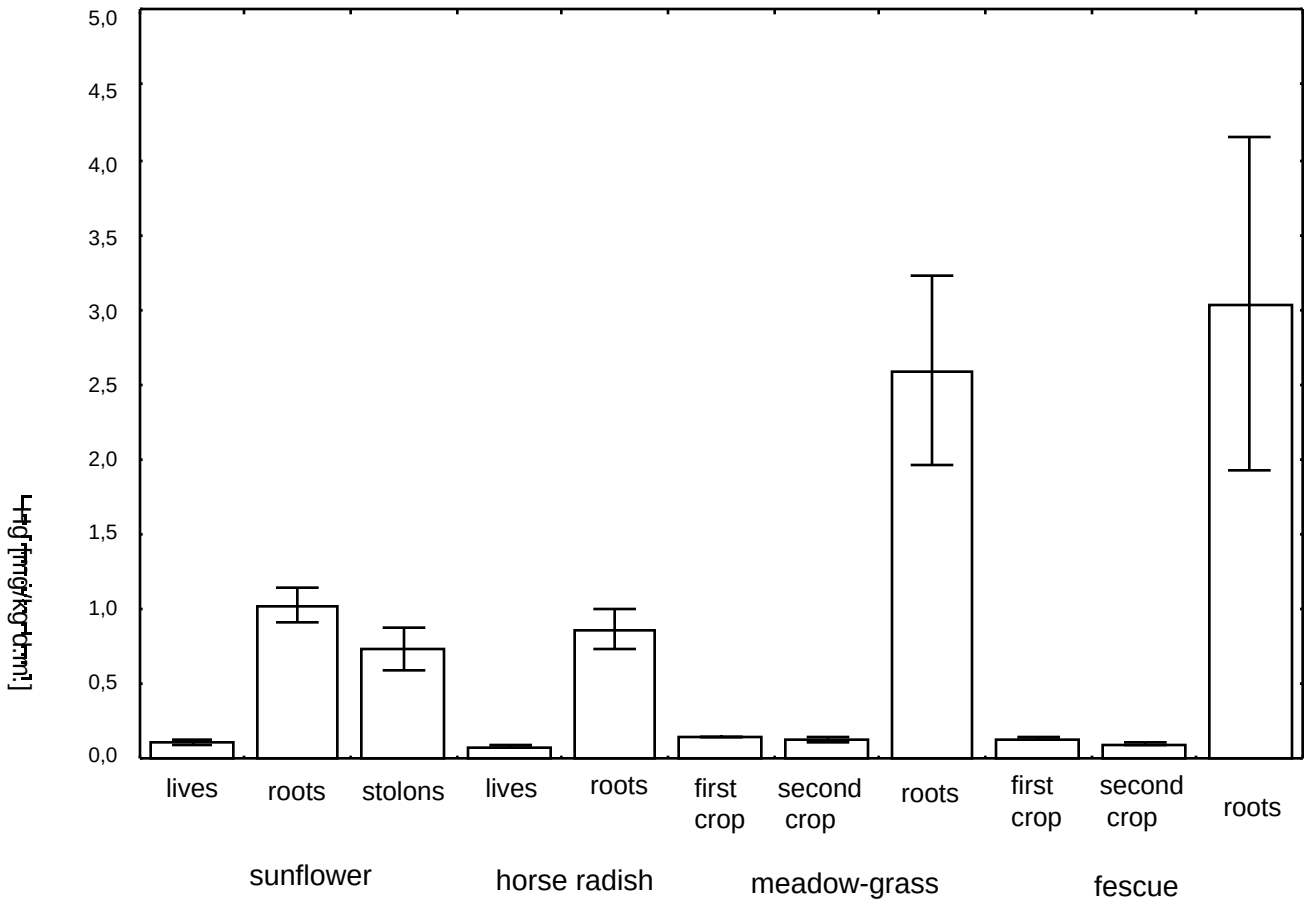

Figure 2. Content of mercury in different plant species and plant parts.

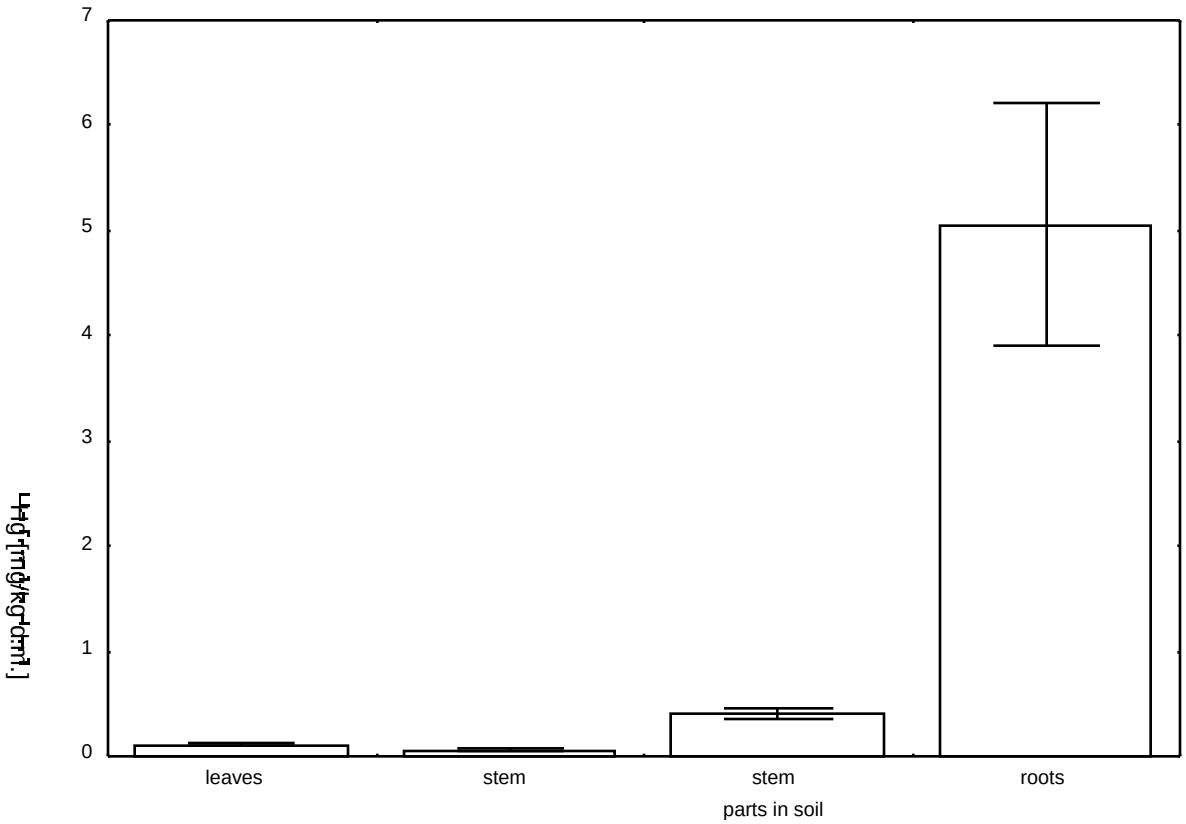

Figure 3. Concentration of mercury in willow (*Salix viminalis*)

Mercury concentration in plants grown on garden soil (Figure 1) ranged from approximately 0.05 to 0.08 mg/kg of dry mass. In the case of sunflower concentrations of mercury were higher (0.17 mg/kg of d.m.-leaves; 0.21-mg/kg d.m.-roots). Sunflower bulbs were collected from plots fertilized four years ago with municipal waste compost, which may be a reason for higher concentrations of mercury in plant tissue.

In all investigated plants, mercury concentration in leaves was lower than in roots and other underground parts of plants (bulbs in sunflower and underground part of willow) (figures 2 and 3). Based on these results, all investigated plants would work well for phytostabilization.

Plant root investigations revealed that grasses (meadow grass and fescue) produced huge root systems with a good soil exploration branch system. Differences between the root system of grasses grown on mercury contaminated and uncontaminated soil were not significant. The opposite effect was observed in the other plant species. Poorer root system architecture was noted on mercury-contaminated soil when compared to the root system architecture of plants grown on garden soil.

Microbiological investigations

Based on the results presented in Table 2 it can be concluded, that the micro-flora in garden soil is richer than the micro-flora in Hg-contaminated soil, except for *Streptomycess* spp. which is similar in both of soils.

Table 2. Microbial characteristic of soil used in experiments [CFU g-1]

	Hg-contaminated soil	**Garden soil**
Heterotrophic soil bacteria	3.26×10^5	3.46×10^7
Pseudomonas	5.59×10^3	7.85×10^5
Streptomycess	1.38×10^5	6.88×10^5
Soil fungi	9.75×10^3	7.76×10^5
Protein-decomposing bacteria	8.04×10^4	7.85×10^6
pH (H_2O and KCl)	6.10 /5.89	5.65/5.38

Microbial characteristics of root-free soil and rhizosphere of ***Poa pratensis*** and ***Festuca rubra*** are presented in Table3 and Table 4. These species of plants also seem to be acceptable for stabilization purposes.

Table 3. Microbial characteristics of root-free soil and rhizosphere soil of *Poa pratensis* (crops 1 and 2) in garden soil and in Hg-contaminated soil [CFU g^{-1}]

	Garden soil			Hg-contaminated soil		
	Rhizosphere (crop 1)	**Rhizosphere (crop 2)**	**Root-free soil**	**Rhizosphere (crop 1)**	**Rhizosphere (crop 2)**	**Root-free soil**
Heterotrophic soil bacteria	3.49×10^8	1.65×10^8	2.57×10^7	4.94×10^8	7.19×10^7	1.22×10^7
Pseudomonas	5.49×10^7	1.53×10^6	1.42×10^6	3.62×10^7	3.15×10^6	1.66×10^5
Streptomyces	2.15×10^7	1.73×10^6	3.42×10^5	4.09×10^6	1.24×10^6	1.55×10^6
Soil fungi	3.05×10^5	9.40×10^4	1.86×10^5	7.98×10^6	7.48×10^4	2.41×10^5
Protein-decomposing bacteria	8.12×10^7	8.70×10^6	2.32×10^7	4.49×10^7	1.71×10^6	9.35×10^6
Nitrifying bacteria	8.34×10^4	2.75×10^4	3.15×10^5	2.75×10^3	3.02×10^3	1.18×10^2
Ammonifying bacteria	1.26×10^4	3.57×10^4	6.65×10^5	3.08×10^2	2.61×10^2	1.08×10^3
Sulfur-amino Acids-decomp. Bacteria	2.13×10^2	1.17×10^2	8.25×10^2	2.05×10^1	2.11×10^1	5.14×10^1

Table 4. Microbial characteristics of root-free soil and rhizosphere of *Festuca rubra* (crops 1 and 2) in garden soil and in Hg-contaminated soil [CFU g^{-1}]

	Garden soil			Hg-contaminated soil		
	Rhizosphere (crop 1)	**Rhizosphere (crop 2)**	**Root-free soil**	**Rhizosphere (crop 1)**	**Rhizosphere (crop 2)**	**Root-free soil**
Heterotrophic soil bacteria	1.54×10^9	6.61×10^8	3.73×10^8	4.48×10^8	2.64×10^8	1.39×10^8
Pseudomonas	9.74×10^8	1.14×10^6	3.35×10^6	1.30×10^7	7.44×10^5	1.52×10^5
Streptomyces	4.46×10^7	3.08×10^6	4.34×10^5	5.56×10^6	8.22×10^6	8.68×10^5
Soil fungi	1.07×10^6	6.02×10^4	1.02×10^5	9.77×10^4	8.47×10^4	3.08×10^4
Protein-decomposing bacteria	1.06×10^9	1.60×10^7	6.87×10^7	7.34×10^7	6.24×10^6	8.99×10^6
Nitrifying bacteria	9.06×10^4	2.84×10^5	4.18×10^5	1.12×10^4	1.81×10^4	4.18×10^3
Ammonifying bacteria	3.16×10^5	5.32×10^5	8.98×10^5	3.84×10^4	4.27×10^4	1.05×10^5
Sulfur-amino Acids-decomp. Bacteria	2.68×10^2	2.33×10^2	4.34×10^2	2.14×10^1	1.26×10^1	3.88×10^1

Table 5. Comparison of the number of ***Pseudomonas*** spp. and sulfur-amino acids-decomposing bacteria of *Poa pratensis* and *Festuca rubra* rhizosphere (crops 1 and 2) in Hg-contaminated soil [CFU g^{-1}]

	Garden soil		**Hg-contaminated soil**	
	Rhizosphere (crop 1)	**Rhizosphere (crop 2)**	**Rhizosphere (crop 1)**	**Rhizosphere (crop 2)**
Pseudomonas	3.62×10^7	3.15×10^6	1.30×10^7	7.44×10^5
Sulfur-amino Acids-decomp. bacteria	2.05×10^1	2.11×10^1	2.14×10^1	1.26×10^1

Table 6. Microbial characteristics of root-free soil and rhizosphere of *Poa pratensis, Festuca rubra* (crops 1 and 2), *Salix viminalis, Armoracia lapathifolia* and *Helianthus tuberosus* in Hg-contaminated soil [CFU g^{-1}]

	Pseudomonas		**Sulfur-amino acids-decomposing bacteria**	
	Rhizosphere	**Root-free soil**	**Rhizosphere**	**Root-free soil**
Poa pratensis				
crop 1	3.62×10^7	1.66×10^5	2.05×10^1	5.14×10^1
crop 2	3.15×10^6	-	2.11×10^1	-
Festuca rubra				
crop 1	1.30×10^7	1.52×10^5	2.14×10^1	3.88×10^1
crop 2	7.44×10^5	-	1.26×10^1	-
Salix viminalis	9.39×10^5	9.95×10^4	1.05×10^1	2.89×10^1
Armoracia lapathifolia	2.87×10^6	1.17×10^5	3.19×10^2	8.12×10^1
Helianthus tuberosus	2.12×10^6	2.13×10^4	5.24×10^1	3.52×10^1

The roots of plants (*Poa pratensis* and *Festuca rubra*) interact with a large number of different microorganisms (e.g. *Pseudomonas, Streptomyces***,** soil fungi, protein-decomposing bacteria). Generally microbial populations were more abundant in the rhizosphere than in the root-free soil. More soil microorganisms inhabited the roots of *Festuca rubra* in the garden soil and in the roots of *Poa pratensis* in the Hg-contaminated soil.

The roots of plants *Helianthus tuberosus* and horseradish also interact with a variety of microorganisms (e.g. *Pseudomonas*, fungi, *Streptomyces***,** protein-decomposing bacteria) but their root system was not explorative as in the case of the grasses. Microbial populations were more abundant in the rhizosphere than in root-free soil.

More soil microorganisms were found in the roots of *Helianthus tuberosus* and horseradish in the Hg-contaminated soil. Only *Streptomyces* spp. were found in the roots of horseradish in the standard garden soil.

A comparison of the number of *Pseudomonas* and sulfur amino-acids-decomposing bacteria of fescue (*Festuca rubra*) and meadow grass (*Poa pratensis*) rhizosphere between mercury-contaminated soil and garden soil is presented inTable.5

There were no differences in CFU (number of colony forming units) in population of *Pseudomonas* and sulphur-amono-acids decomposing bacteria between crop one and two and only small differences between garden soil and mercury-contaminated soil.

Microbial characteristics of *Pseudomonas* and sulfur-aminoacids- decomposing bacteria in the root-free zone of mercury-contaminated soil and rhizosphere of all investigated plants are presented in Table 6.

These results show higher numbers of *Pseudomonas* in the rhizosphere of grasses.

Conclusions

- Plant growth and development on mercury-contaminated soil was comparable to the reference garden soil.
- Investigated plants accumulate mercury compounds mainly in their roots.
- The highest amounts of mercury were found in willow (*Salix viminalis*) roots; however, a willow's root system is located mainly in the upper layer of mercury-contaminated soil, which is not helpful in stabilizing these soils.
- Both investigated grasses (meadow grass and fescue) accumulate less mercury than willow, but created a good soil penetration (i.e.,stabilization) system.
- The highest numbers of *Pseudomonas* were found in the rhizosphere of both grasses and willow. The number of *Pseudomonas* was higher in the rhizosphere when compared to the root-free zone. Most likely *Pseudomonas* development was supported by special root excretion.
- Mercury-contaminated soil microflora investigations demonstrate the variability among physiological groups and taxons of bacteria. The soil, although contaminated with mercury, does promote plant growth.
- The plants have developed a rich rhizosphere zone in mercury-contaminated soil. At the same time plant's roots have appeared to be mercury excluders. Both phenomena are essential for soil stabilization processes using plants.