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PETROLEUM CONTAMINATED SOILS BIOREACTOR: OPTIMIZATION AND ENHANCEMENT

Executive Summary

The project Production Scale Implementation of a Petroleum Contaminated Soils Bioreactor was launched in FY00 and showed that bioremediation can be performed under strictly controlled conditions (Worsztynowicz, 2000). Initial results showed a 50% reduction of total petroleum hydrocarbon concentration in soil from the Czechowice-Dziedzice refinery. During the 97 days of the experiment TPH content in soil was reduced from ~24 g/kg of soil to ~12 g/kg of soil. The systems, which controlled and monitored the bioreactor were tested and found to be satisfactory. However, it was determined that changes in the design and construction of the bioreactor could improve system performance.

In FY01, the bioreactor design was modified to increase bioremediation effectiveness, simplify maintenance procedures, improve equipment utilization and prepare for process automation. Modifications included changes in the leachate recirculation system, improvement of the bioreactor s thermal insulation and changes in the data acquisition system. Once these adjustments were made, two operational campaigns (tests) were conducted to determine which operating parameters optimized performance.

During the testing phase, the following parameters were monitored continuously in either the soil or the headspace of the system: oxygen, carbon dioxide, hydrocarbon concentration, air humidity, air flow rate, and liquid level and flow rate. Periodically, leachate and soil samples were collected to determine hydrocarbon concentrations and microbial activity, and respiration tests were done to help determine system kinetics. The results show big reduction of gasoline (from ~800 mg/kg to 13.1˚mg/kg). The biodegradation of TPH in case of diesel oil was also observed, but the results are much more difficult to interpretation.

1. Introduction

Petroleum contaminated soils and sediments 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 on-site, batch, *ex situ* remediation. This approach also has been proposed for removing organic contaminants from mixed waste (hazardous waste with a low-level radioactive component), thus allowing the waste to be disposed of as a low-level waste, a much simpler situation than disposal of mixed waste. This technology deals well with comparatively small volumes of waste material and supports relatively stringent clean-up standards. Highly controlled, small-scale operations seem well suited to such an activity and could be used widely throughout the DOE complex and in other locations worldwide.

Bioremediation is a proven technology for removing organic contaminants from soil. It is a process that mineralizes or transforms hydrocarbons (both xenobiotic and naturally occurring) that released into the environment into less toxic or innocuous forms (Alexander, 1999). Many microorganisms, including bacteria, fungi, yeast and algae, have the enzymatic capacity to completely mineralize petroleum hydrocarbons and utilize the carbon component to generate new biomass. Indigenous microorganisms in the soil and groundwater can degrade large quantities of petroleum hydrocarbons when provided sufficient amounts of water, oxygen, and nutrients such as nitrogen and phosphorus.

The mobile, packed soil bed, continuous air flow bioreactor was built by the Institute for Ecology of Industrial Areas (IETU) based on a design supplied by the Westinghouse Savannah River Company (WSRC), Aiken, SC (Kastner, 1998). The bioreactor was built to demonstrate the possibility of bioremediating hydrocarbons in strictly controlled conditions would increase technology capabilities and could be used with highly contaminated soil. Its design incorporated knowledge gained during execution of the biopile project at the Czechowice Oil Refinery (CzOR). The system operational capabilities were examined during Startup tests (Worsztynowicz, 2000) with promising results. However, some modifications to the design and construction of the bioreactor were called for, in order to increase its effectiveness and easy operation.

The objective of this project was to redesign the unit and modify its construction in order to increase the effectiveness of the bioremediation process and to facilitate maintenance of the bioreactor. Technology enhancements also were incorporated, including the use of inoculum. The effectiveness of the modifications and technology enhancements was analyzed during two separate bioremediation tests.

2. Bioreactor system modifications and enhancements

2.1 Leachate Recirculation System

The leachate recirculation system was modified to increase its capacity, improve system sampling, and improve operation of the leachate recycling system. T-type connecting

junctions and additional valves were mounted on the pipes between the leachate tank and the pump, and between the pump and the bioreactor vessel. The junctions and valves, added between the leachate tank and the pump helped with pump maintenance (particularly when the tank needed to be filled with clean water). The valves and junctions, added between the leachate pump and the bioreactor vessel provided an easy method for sampling and emptying the system. This was especially useful when soil rinsing was needed.

2.2 Bioreactor Thermal Insulation

Bioreactor soil temperature measurements provided evidence that ambient weather conditions had a significant effect upon soil temperature inside the bioreactor. This was especially true during winter months, when the rate of bioremediation decreased significantly. The impact of changing weather conditions also was observed when there was a large difference in daytime and nighttime temperatures. The addition of thermal insulation was necessary to reduce the impact of the weather on bioreactor performance.

The walls, lid and floor of the bioreactor were covered with a layer of plastic. Due to its low thermal conductance, styrofoam was chosen as insulation and was fixed to the inside of the bioreactor vessel. Styrofoam is not resistant to organic compounds (e.g., hydrocarbons), so it was covered with a coat of paint. This configuration kept the soil from coming into contact with the metal wall, thus reducing heat loss due to conduction. It also provided a layer of protection to the bioreactor wall, hence avoiding or reducing the corrosion that was evident after initial testing in FY00. A diagram of the thermal insulation system is presented in Figure 1.

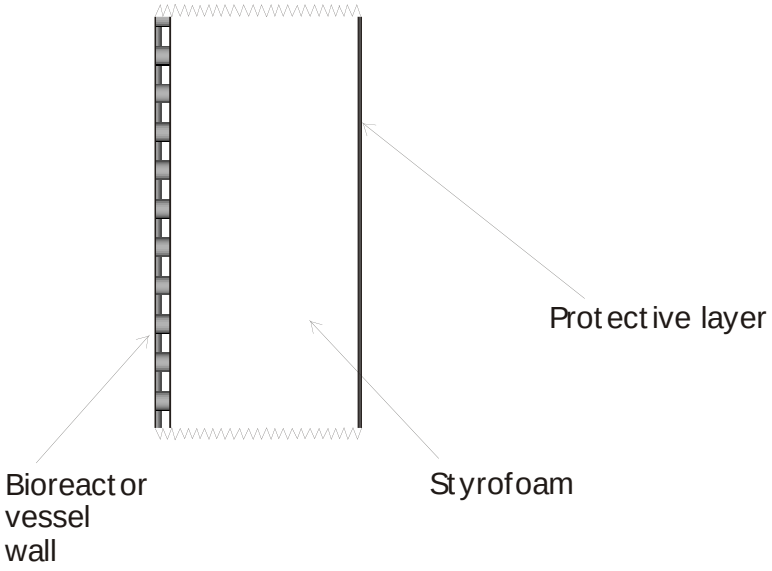


Fig 1. Schematic of the bioreactor thermal insulation

2.3 Bioreactor Monitoring System

2.3.1 Sensors

The bioreactor monitoring system consisted of five sets of sensors:

— three sensor sets mounted in the soil bed. All sensor sets were placed horizontally, in the middle of the soil layer, along the bioreactor axis. Horizontal sensor position prevented the migration of outlet gases into sensor chambers. The sets contained thermocouples and hydrocarbon sensors. Two carbon dioxide sensors also were mounted in the soil bed;

— one sensor set mounted at the gases outlet. This set contained sensors for oxygen, hydrocarbons, carbon dioxide and a thermocouple sensor; and

— one reference set mounted at the air inlet. This set included an oxygen sensor, hydrocarbon sensor and thermocouple.

Additionally, inlet and outlet gas volumes, as well as humidity, temperature and pressure were monitored. Sensors placement in the bioreactor is shown in Figure 2.

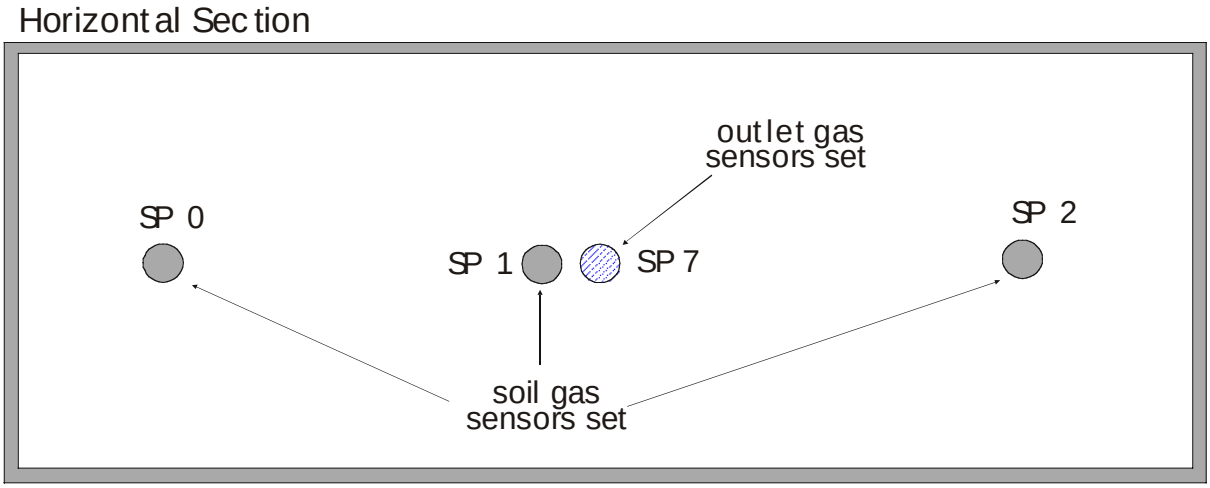


Figure 2. Sampling port placement in the bioreactor

A set of electrical connectors was placed between ADAM modules and the bioreactor vessel. They were placed in two airtight, cast iron boxes and mounted on the bioreactor wall. All equipment installed inside the vessel was connected by mounting clips to the data acquisition modules (Figure 3). Thus, installation and connection of different sensors were highly simplified.

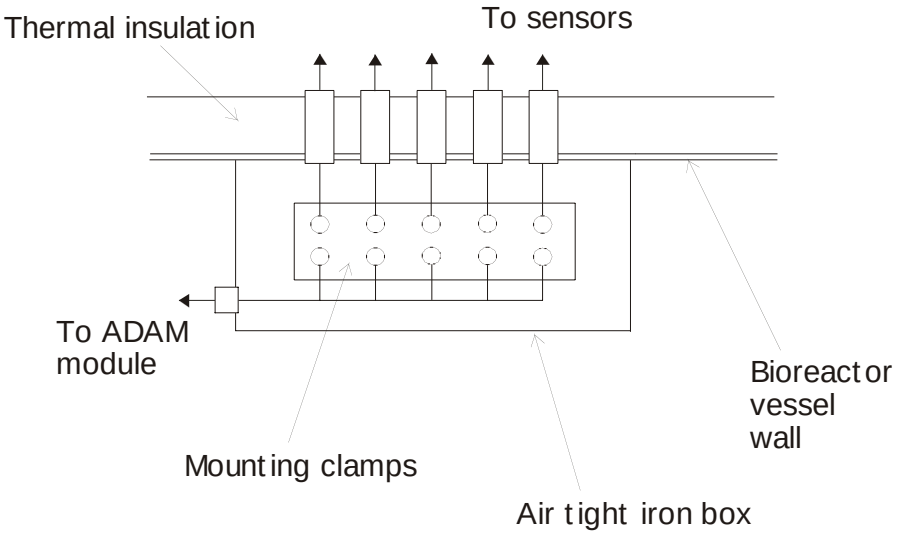


Figure 3. The electrical connectors set between sensors and ADAM modules

An electrical energy meter, which allows for a better estimation of the process costs was installed to measure energy consumption during the bioremediation test.

2.3.2 Data acquisition and processing software.

Data acquisition software was redesigned to reflect changes in sensor wiring design, because the TCE bioremediation project also needs automated monitoring. The software was enhanced so that data acquisition from the TCE bioreactor could be performed from the same application. Software modifications included the following:

_ enhancing capabilities during measurements,

_ adapting to new sensor configuration,

_ creating a new user interface,

_ developing new data logging procedures, and

_ adding a module for TCE bioreactor data acquisition.

All software was created using the Advantec VisiDaq computer software. The MS Access database for measurements, data storage and processing was designed and implemented. Data stored in the database can be exported to MS Excel or other software for further analysis or presentation.

3. Bioremediation Tests

Two operating campaigns were planned for FY01. The first campaign continued to focus on optimizing bioreactor operation and on the development of mathematical bioreactor models. The second test phase incorporated lessons learned during the first campaign and focused on treatment of material more difficult to biodegrade. System changes and process enhancements were monitored to determine their impact on bioreactor operation.

3.1 Bioreactor System Preparation

3.1.1 Vessel unloading

After removal of the monitoring and controlling equipment, the vessel was returned to the Czechowice—Dziedzice Refinery. The remediated soil was unloaded without difficulty

3.1.2 Bioreactor system maintenance

After soil unloading, all systems within the bioreactor, as well as the vessel itself, were examined for faults. Subsystems were taken apart, tested and reassembled.

Gas and temperature sensors were tested for accuracy in ambient air. The oxygen sensor was disassembled and repaired. All electrical wiring was tested and checked for signs of mechanical faults. Some corrosion rings were observed inside the bioreactor vessel. The corrosion was removed and the inner surface of the vessel was cleaned

and covered with anti-corrosive paint. The aeration and leachate recirculation subsystems were checked for faults. The bioreactor vessel and subsystems were tested for air tightness.

3.1.3 Soil preparation

Soil was contaminated artificially with gasoline and diesel oil for bioreactor operational campaigns in FY01. First the soil was excavated from the IETU yard. All stones and large solids were removed. The soil was sieved and mixed with wood chips. Approximately 0.6˚m^3 of pine wood chips were mixed with 3 Mg (metric tons) of soil to loosen the soil and allow for easier aeration. The soil was loaded into the bioreactor vessel and appropriate amounts of gasoline (~3˚kg) or diesel oil (~7.2 kg) were added. Efforts were made to get an even distribution of contaminants in the soil, to reduce exposure to toxic compounds and to lower the fire danger. The initial concentrations of gasoline and diesel oil, calculated on added amounts of contaminants, were˚~1000˚mg/kg and ~2000˚mg/kg respectively. Soil was sampled to estimate biological activity.

3.1.4 Inoculum preparation

Microorganisms (bacteria and fungal strains) were isolated in previous experiments, and stored at —20°C in glycerol (35% v/v): saline (0.85% w/v). From the experiment 3 isolated bacterial strains (*Shingomonas paucimobilis, Stenotrophomonas maltophilia, Pseudomonas fluorescens*) were characterized well for their ability to degrade hydrocarbon compounds (unpublished results). These strains had the highest biodegradation rate, and then were used in inoculum preparation.

To obtain pure bacterial strains, 100 µL of cell suspensions were put into 50 mL of liquid medium composed of 8 g peptone, 2.5 g yeast extract and 1 g glucose. All incubations were conducted at room temperature with shaking at 150 rpm for 2 days. The composition of inoculum was as follows:

- mineral salts (g/liter): NH_4NO_3 — 1 g; K_2HPO_4 — 1 g; KH_2PO_4 — 1 g; $MgSO_4 * 7\ H_2O$ — 0.2 g; $CaCl_2 * 6\ H_2O$ — 0.02 g; $FeCl_3 * 6\ H_2O$; 1 mL of trace solution (biostimulation), and

- bacterial cells of *Shingomonas paucimobilis, Stenotrophomonas maltophilia, Pseudomonas fluorescens* (bioagumentation).

Two soil types (soil used in the bioreactor and soil from the refinery) were examined.

3.2 Bioremediation Task 1

Bioremediation Task 1 was designed to:

- ensure the effectiveness of bioreactor equipment for remediating light hydrocarbons,

_ test the performance of process monitoring equipment (new architecture and hydrocarbon sensors),

_ assess the influence of process enhancements on bioremediation effectiveness, and

_ determine the usefulness of bioreactor system modifications.

In addition to these subtasks, data analyses recorded during bioremediation Task 1 could be helpful in creating a mathematical model for the process.

To begin Task 1, the soil surface inside the bioreactor vessel was sprinkled with an emulsion of water and gasoline. Approximately 3 kg (4 dm^3) of unleaded gasoline was used for soil contamination. After hydrocarbon addition, data acquisition was started and the bioreactor was left without aeration for 5 days. Sensor responses were logged into a text file, then exported into MS Access and MS Excel for further analysis. A microbial inoculum was added to the leachate recirculation tank and pumped onto the contaminated soil in the vessel. This method ensured an even distribution of inoculum in the remediated soil. Soil samples were taken periodically for laboratory tests. Results of the analyses are shown in Table 1 and Figure 4. SP is the abbreviation of Sampling Point.

Table 1. Gasoline concentrations in soil [mg/kg]

Date	**Sampling point**		
	0	**1**	**2**
07/09/2001	101	108	144
07/20/2001	14.4	21.5	15.7
08/16/2001	17.0	10.5	11.9

The obtained data showed that the concentration of gasoline contaminated soil dropped from approximately 800 mg/kg to less than 20 mg/kg (a value recommended by the Polish State Inspectorate for Environmental Protection) in about 6 weeks.

Sensors measured soil gas composition, as well as soil temperature. Although most of the measurements went smoothly there were a few problems to consider. A detailed performance description of all sensor groups as well as their type and manufacturer information is presented in Table 2.

Figure 4. Gasoline concentrations in soil [mg/kg]

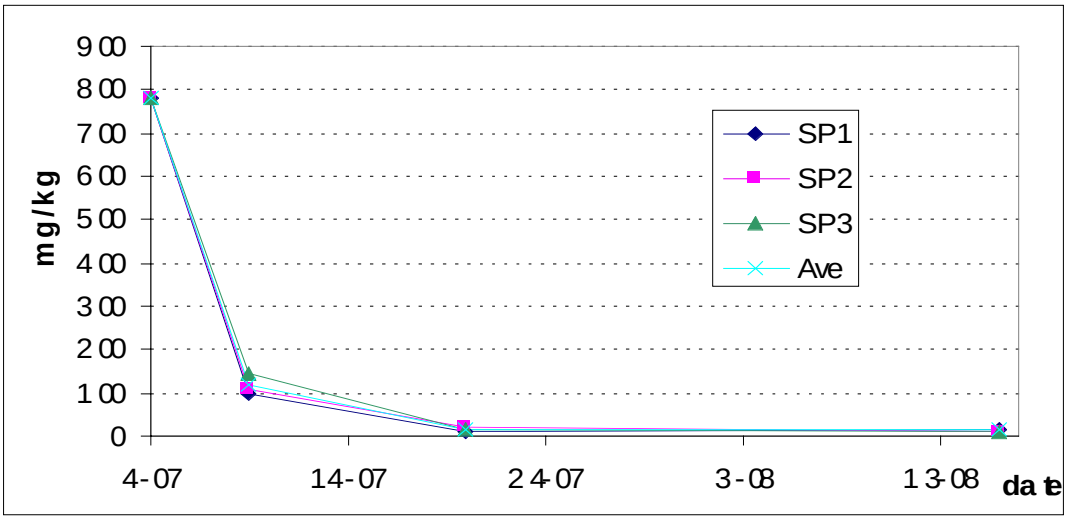


Table 2. Types of sensors used

Measured parameter	Sensors type	Supplier	Description
Oxygen contents	DRC-XT253	Microbac US	O_2 contents in soil and outlet gases
Hydrocarbons contents	DRC-ADS201	Microbac US	Hydrocarbon (HC) contents in soil and outlet gases
Carbon dioxide contents	GMM220	Vaisala US	CO_2 contents in soil and outlet gases
Soil temperature	DRC-TKO24	Microbac US	Thermocouple type K
Gas humidity and temp.	EE-15	Introl PL	Measure of inlet and outlet gases
Gas flow meter	BK-6	Intergaz PL	Measure of inlet and outlet gases
Gas pressure	Eco-tronic	Introl PL	Measure of inlet air pressure

O_2 — Two oxygen sensors were mounted in the bioreactor system: on the air inlet and outlet. The outlet gas sensor was mounted inside the bioreactor vessel, beside the outlet pipe. Both sensors worked smoothly. Observed oxygen levels were 21% in inlet air and, approximately 18% in outlet gases. Sensor characteristics were noted during the Startup Test (FY00) and corrected if necessary. Oxygen sensors were mounted

vertically. Because of their relatively low output signal (~30˚mV), all connections were made of shielded two wire cable. Output signals are of voltage nature (DC), so they were easy to measure and did not require additional circuits. Oxygen sensors did not display any signs of cross sensitivity to hydrocarbons (gasoline). This type of sensor is recommended for future applications.

CO_2 — Three carbon dioxide sensors were installed: one on the effluent gas line and two in the soil. Observations showed a typical concentration of about 2.5%. The sensors did not require any characteristic corrections; they were accurate and stable. Although the sensors do require a power supply to operate, their application is easy. Attention must be paid to avoid installing the CO_2 sensors next to thermocouples or other sensors, which are sensitive to heat, as they are warm during measurement. This type of sensor is recommended for future applications.

Hydrocarbons — Five hydrocarbon sensors were installed: one set on the air inlet, one set on the gas outlet and three sets inside the soil bed. After gasoline was added to the soil, sensor output signals increased from values typical for ambient air to maximum voltage supplied by a constant current source. Sensor resistance dependence on hydrocarbon concentration can be expressed by the following formula:

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

where R_0 is resistance in ambient air, c is hydrocarbon concentration and k is a calibration constant. In case of high sensitivity, sensor resistance can change from ~0.5 kΩ (typical for ambient air) to ~1 MΩ, which is far too much for recording by means of ADAM modules and constant current set. Correction of sensor characteristics can be achieved by mounting a set of ~10 kΩ resistors parallel to sensor outputs. This limits the maximum measurement channel resistance while ensuring accurate measurement of low concentrations (of hydrocarbons). However, it is still necessary to adjust constant current output values. Addition of a parallel resistor has to be considered in sensors channel output voltage dependence on hydrocarbon concentrations. After calculations, sensor resistance dependence on hydrocarbon concentration can be expressed by the following formula:

$$R = \frac{R_0 R_p}{R_0 + R_p \text{?} 10^{-k'c}}$$

where R_p is resistance of an added parallel resistor. Since exact calibration of the sensors cannot be done in the case of gasoline, hydrocarbon sensor output signals were treated as reference values and were used to determine bioremediation progress. Unfortunately, the hydrocarbon sensors still were unstable and unreliable. After bioremediation Task 1 the sensor response to clean air was different from the response at the beginning of the test. This type of sensor is not recommended for future applications.

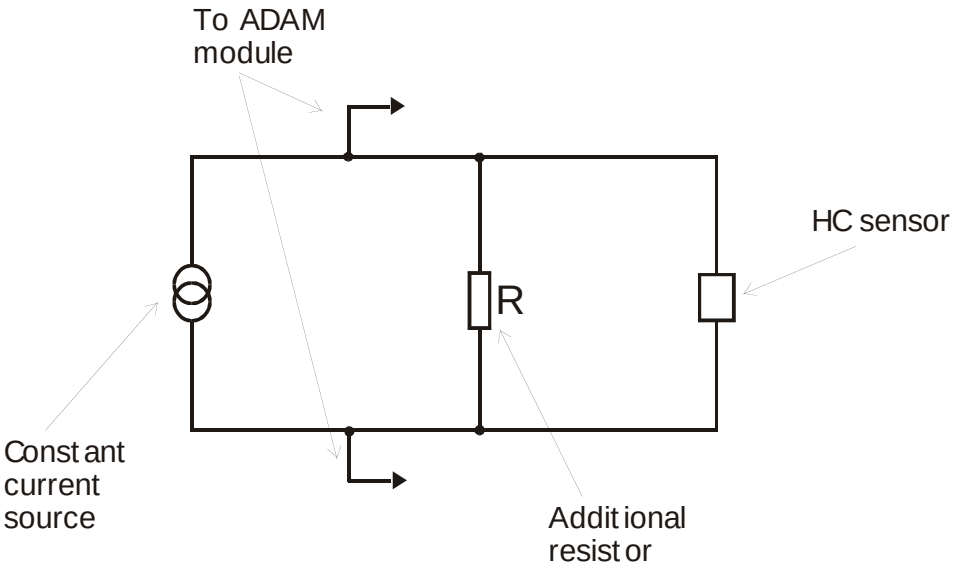


Figure 5. Modified hydrocarbon sensor measurement channel

Temperature — Four thermocouples were installed in the bioreactor vessel: three in the soil bed and one set on the gas output. Since hydrocarbon sensors are temperature dependent, thermocouples were mounted on the hydrocarbon sensor housing, thus allowing for temperature compensation. It is important to use a compensation cable, specifically selected for a given type of thermocouple, for the thermocouple connection to ADAM modules. Attention must be paid to proper thermocouple polarization. K type thermocouples, connected with Teflon coated cable were more accurate and stable and can be used successfully in other bioremediation applications.

Measurements performed during the bioremediation experiment indicate the following:

_ thermal insulation of the bioreactor vessel helped to stabilize bioremediation conditions. Soil temperature differences between day and night were much lower than observed in the start-up task without insulation;

_ verification of sensor functioning and accuracy was essential, due to the potential for failure. Though every sensor was tested carefully before mounting inside the vessel, one of them did not work properly during the experiment. Special attention has to be paid to this aspect during bioreactor system automation. It is suggested that at least 2 sensors of a given type should be mounted inside the bioreactor vessel;

_ boxes with mounting strips are helpful in sensor configuration changes and quick sensor replacement;

_ sensor measurement results are qualitatively consistent; and

_ signs of corrosion may be observed on styrofoam used for thermal insulation, despite the application of a protective coat of paint. Mounting thermal insulation on the outside layer, though more difficult, is recommended.

Results of measurements are presented in Figure 6.

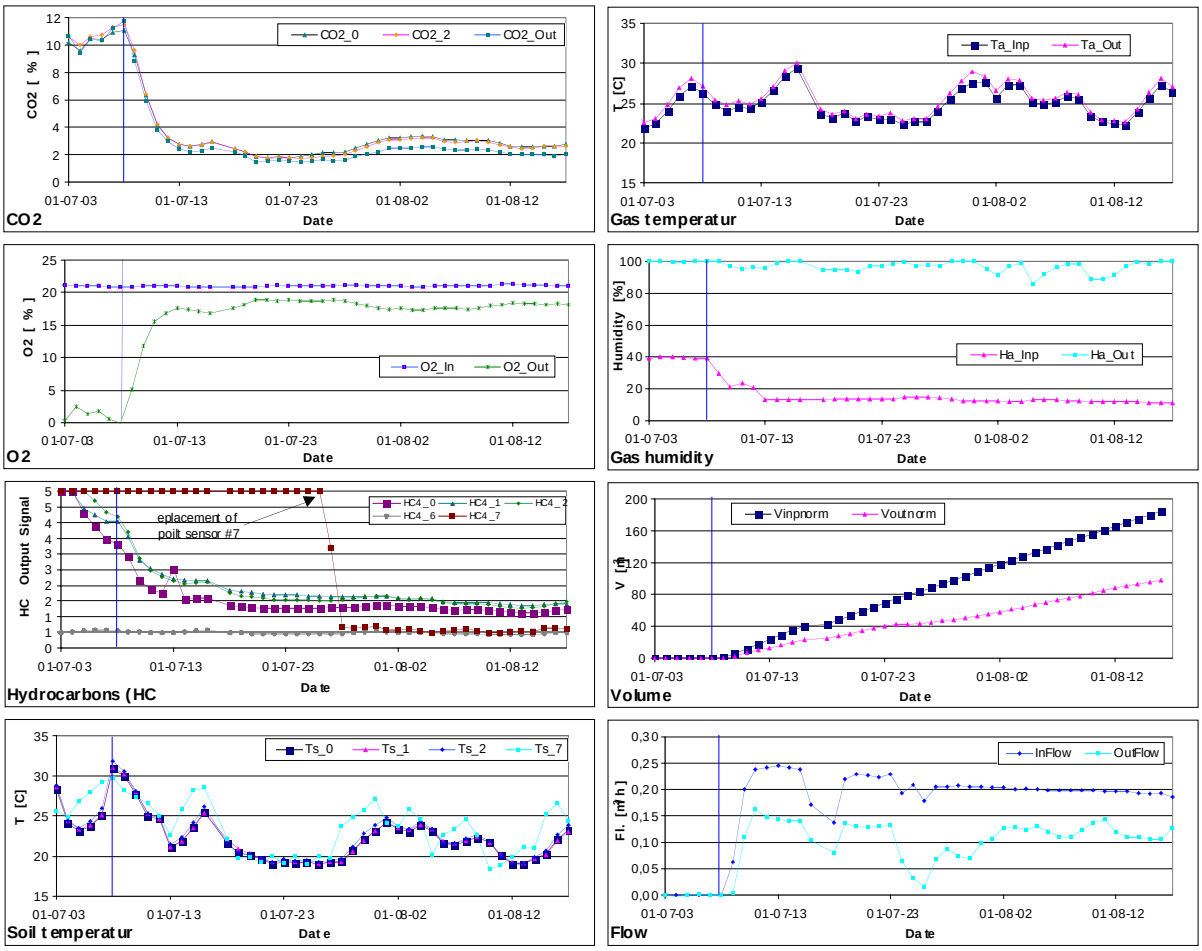


Figure 6. Sensor measurement data from Task 1

3.3 Bioremediation Task 2

Bioremediation Task 2 was designed to ensure bioreactor system performance in soil contaminated with heavier hydrocarbons. Summer diesel oil was chosen as a contaminant. Specifically, Bioremediation Task 2 was designed for the following:

- test the performance of process monitoring equipment, particularly readout changes or difficulties which may appear with a different contaminant type;
- assess the influence of process enhancement (bioaugmentation) on bioremediation effectiveness;
- check the usefulness of bioreactor system modifications; and
- gather data to help estimate a mathematical model of the process.

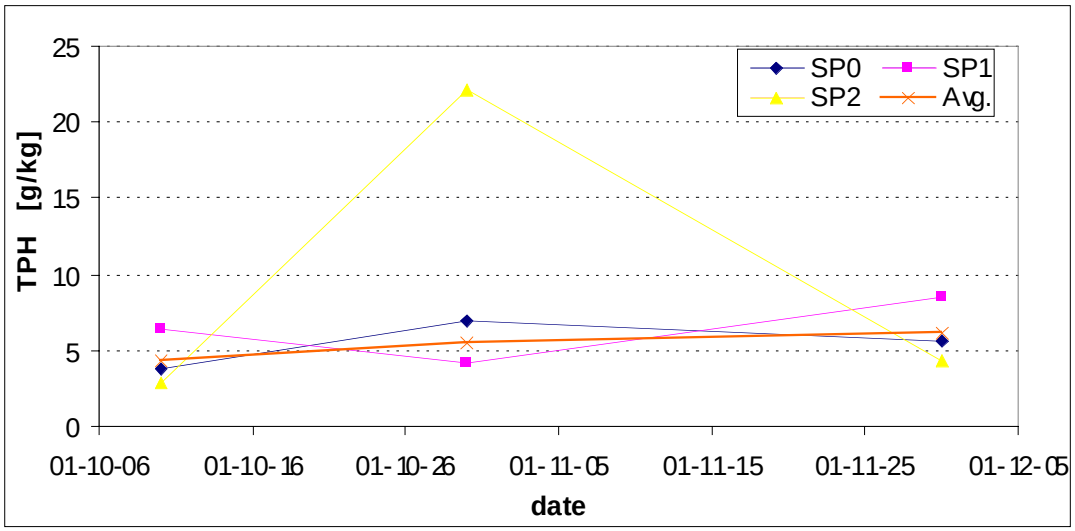
The soil surface in the bioreactor vessel was sprinkled with approximately 7.2 kg (9˚dm^3) of summer diesel oil. After hydrocarbon addition, data acquisition was started. Sensor responses were logged into a text file, then exported to MS Access and MS Excel for further analysis. Soil samples were collected periodically for laboratory tests.

The bioreactor was left without aeration for a few days to achieve equilibrium in contamination distribution due to their diffusive transport. Because of lower temperatures and less volatile hydrocarbon, the aeration system was turned on after 20 days. The soil samples were taken periodically for analysis. The measured TPH concentrations are presented in Table 3. There was neither inoculation nor surfactants added to the system during the test.

Table 3. TPH concentrations in soil [g/kg]

Date	Sampling point		
	0	1	2
10/10/2001	3.85	6.40	2.86
30/10/2001	6.90	4.13	22.1
30/11/2001	5.64	8.49	4.30

Figure 7. TPH concentrations in soil [g/kg] (Task 2)



Results gained during analyses show that not only TPH concentrations increase in time during process but also they are much greater than one could expect. The maximum hydrocarbon concentration, calculated on added amount basis, is 2.4 g/kg. Differences between calculated and measured values can be explained in two ways:

- samples taken for analysis had extremely high TPH concentration due to uneven distribution of diesel oil in soil. This can explain high TPH concentration in SP2 sample from 10/30/01, which was omitted in calculated averages. However, it is unlikely that all samples expose the same kind of systematic error and tends to increase in time.

- There is an additional source of hydrocarbons in the bioreactor vessel. This explains increasing values of TPH during experiments as well as TPH concentration higher than the expected maximum.

It is likely that styrofoam, used as thermal insulation, dissolves during the experiment and serves as additional source of hydrocarbons. The corrosion signs (despite the protective layer) were observed during Task 1 experiments. However, the greatest area of styrofoam exposed to hydrocarbons is under the soil surface and can be checked after the bioreactor unloading. As a conclusion it may be stated that thermal insulation must be mounted outside the vessel. The observed concentration ratio between O_2 and CO_2 shows that the biodegradation process runs but the amount of hydrocarbons released from styrofoam masks its effect.

Soil gas composition, as well as soil temperature, were measured using sensors. The sensor configuration was the same as in bioremediation Task 1. There were no major problems with the data acquisition system. The performance of all sensor groups is presented below, along with recommendations for future applications.

O_2 — Both sensors work smoothly. Observed oxygen level in inlet air was 21 %. Oxygen concentration in outlet gases dropped to 0.5 % during the initial phase (aeration switched off) and then increased to approximately 19 % with aeration. The oxygen sensors displayed no signs of cross sensitivity to the hydrocarbons (diesel oil). This type of sensor is recommended for future applications.

CO_2 — All sensors worked smoothly, without any difficulties. Observed concentrations increased to approximately 9 % during the initial phase of the project, and decreased to approximately 1 % after aeration began. The sensors did not require any special corrections; they were accurate and stable. No hydrocarbon cross sensitivity (neither gasoline nor diesel oil) was detected. This type of sensor can be recommended for future applications.

Hydrocarbons — Because of expected high changes in sensors resistance, a set of resistors (~10 kΩ each) was installed and tested successfully. However, sensor sensitivity to diesel oil turned out to be much lower than for gasoline. Only the outlet gas sensor significantly increased its resistance. Sensors in the soil bed changed their resistance only slightly. To test the influence of the sensor set housing on the readouts, another hydrocarbon sensor was placed directly in the soil bed, without a protective layer made of a geomembrane. Results showed that in the case of less volatile hydrocarbons, (e.g., diesel oil) the geomembrane had a considerable influence on sensor performance. Therefore, it is highly recommended that sensors be placed directly in the soil when bioremediating heavier hydrocarbons. Unfortunately, this

enforces vertical position of the sensor, which may result in the migration of outlet gases into sensor chambers (through spaces along the cable and housing). As stated in the description of bioremediation Task 1, exact calibration of the sensors could not be done, and the hydrocarbon sensor output signals were treated as reference values used to determine process progress. Also, another sensor failed and had to be replaced.

Temperature — Thermocouple sensors were connected to ADAM devices through a compensating cabling. The sensors were accurate, stable and worked well. They can be used in any other bioremediation applications.

Measurements performed during bioremediation Task 2 show the following:

_ Thermal insulation of the bioreactor vessel helped maintain stable bioremediation conditions. Temperature decreases on cold days were significantly less;

_ Thermal insulation must be mounted outside bioreactor vessel. Though it is technically more complicated and more expensive, it prevents soil from being contaminated with hydrocarbons released from styrofoam.

_ Hydrocarbon sensor response to different types of hydrocarbons (different but relatively consistent) can vary significantly. Thus, the sensors have to be tested with a given type of contaminant to determine proper configuration of the measurement channel and to assess the expected range of resistance changes;

_ Housing hydrocarbon sensor within chemical-proof fabric should be avoided in the case of low volatile hydrocarbons as it can influence sensor performance;

_ Sensor measurement results are qualitatively consistent; and

_ Diesel oil, because of its lower volatility, is much easier to handle in the bioreactor than is gasoline.

Results of measurements are presented in Figure 8.

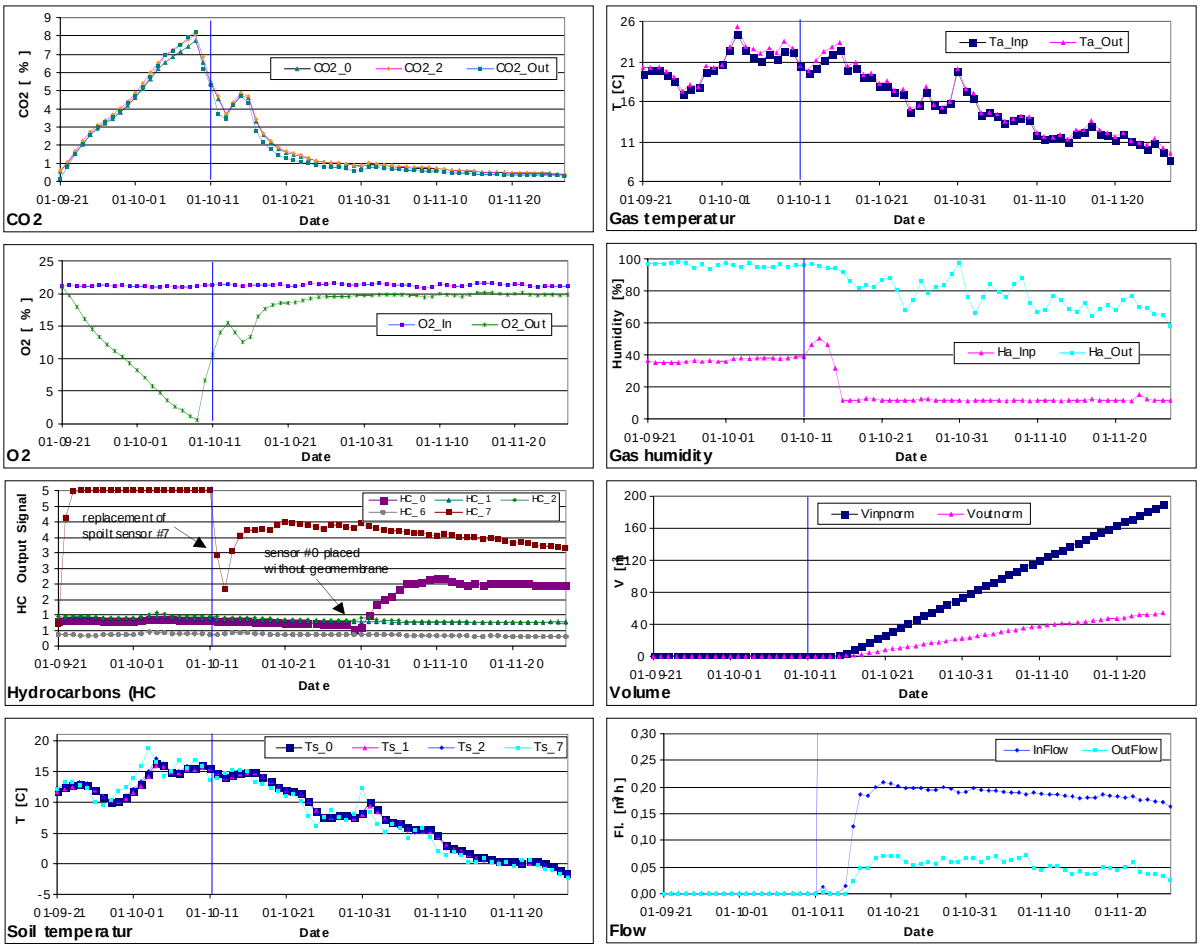


Figure 8. Sensor measurement data from Task 2

4. Conclusions

The bioremediation test carried out in FY01 showed the bioreactor could be used for remediating light hydrocarbon contaminated soil. Implemented equipment modifications worked well and proved useful. The following may be concluded:

- the bioremediation process is fast and can be enhanced by introducing inoculum to the system;

- thermal insulation of the bioreactor vessel helps in acquiring stable bioremediation conditions. Temperature differences between day and night are much lower than observed in the start-up task without insulation. Temperature decreases within the bioreactor on cold days, therefore, the bioremediation rate is significantly lower;

- thermal insulation must be mounted outside bioreactor vessel. Though it is technically more complicated and more expensive, it prevents soil from being contaminated with hydrocarbons released from styrofoam;

- signs of corrosion were observed on styrofoam used for thermal insulation, despite the application of the protective layer of paint. Mounting thermal insulation on the outside of the bioreactor is recommended even though it may be more difficult;

- boxes with mounting strips expedite sensor configuration changes and sensor replacement;

- verification of sensor functioning and accuracy is essential due to the potential for failure. Though all sensors were tested carefully before mounting inside the bioreactor vessel, some of them did not work properly during the experiment. Special attention has to be paid to this aspect during bioreactor system automation. It is suggested that at least 2 sensors of a given type be mounted inside the bioreactor vessel;

- hydrocarbon sensor response to different types of hydrocarbons (different but relatively consistent) can vary significantly. Thus, sensors must be tested with a given type of contaminant to properly configure the measurement channel and to assess the expected range of resistance changes;

- housing hydrocarbon sensors within chemical proof fabric should be avoided in the case of low volatile hydrocarbons as it can influence sensor performance;

- due to problems with accuracy and reliability, choosing different types of hydrocarbon sensors for future applications are recommended;

- oxygen sensors, carbon dioxide sensors and thermocouples worked well. No signs of cross sensitivity were detected. It seems reasonable to use the same type of sensors in the future;

- sensor measurement results were qualitatively consistent;

- modified data acquisition software worked smoothly and allowed comfortable data logging. However, some limitations of the Advantech VisiDAQ environment were observed, including an inability to automatically start data logging after accidental power loss. Though it is not a big disadvantage in a system strictly controlled by humans, it would be unacceptable in an automated system. Therefore, developing new software, running as a Windows NT Service (a system process on Windows NT server) is highly recommended for future applications; and

- diesel oil, because of its lower volatility, is much easier to handle in a bioreactor than gasoline.

5. References

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6. Appendix

The following table contains metal concentrations for soil used in bioremediation Task 1 and Task 2.

Metal	Sampling point			
	SP0	SP1	SP2	Avg.
Cd	4.36	4.12	5.49	4.66
Pb	113.80	112.50	131.40	119.23
Zn	487.40	477.40	558.60	507.80
Cu	18.67	20.60	17.61	18.96
Ni	16.03	13.33	13.84	14.40
Cr	20.74	21.20	23.68	21.87
Co	4.23	4.64	5.13	4.67
Hg	<0.42	<0.42	<0.42	<0.42
As	7.96	7.82	14.41	10.06

USE OF A BIOREACTOR TO TREAT CHLORINATED SOLVENT-CONTAMINATED SOILS

TABLES

FIGURES

Executive Summary

This project was designed, managed and implemented under the direction of the Institute for Ecology of Industrial Areas (IETU) and the Westinghouse Savannah River Company (WSRC) for the United States Department of Energy (DOE). WSRC provided technical support and served as the IETU's customer while Florida State University provided project management support to the IETU. All parties cooperate under DOE EM-50 Joint Coordinating Committee for Environmental Systems (JCCES) agreement.

The initial success of the petroleum-contaminated soils (PCS) bioreactor during FY00 at the IETU has generated interest in applying this technology to other contaminants of interest within DOE. As a result, the application of a second bioreactor to perform chlorinated solvent contaminated soils bioremediation was proposed.

The goal of the FY01 study was to develop a technology for bioremediation of chlorinated solvent contaminated soil using a small, mobile bioreactor. The system was intended to treat trichloroethylene (TCE) contaminated soils but could be used to treat other organic contaminated soils as well. Laboratory tests and bioremediation in the bioreactor were carried out under anaerobic conditions, although the bioreactor has the ability to operate aerobically or anaerobically in batch mode. The project includes the following tasks:

1. treatability study to define optimal parameters for TCE biodegradation;

2. bioreactor design and construction; and

3. bioremediation test of TCE contaminated soil in the bioreactor.

Data from the treatability study provided information, which served as a basis for determining the physico-chemical and microbiological parameters for the remediation of TCE-contaminated soil in a bioreactor. It was found that bacteria present in the sludge mixture are capable of complete dechlorination of TCE to ethylene (ETH) in anaerobic conditions.

The chlorinated solvents contaminated soil bioreactor (CSCS bioreactor) was designed and built in Poland using lessons learned during design, construction and operation of the PCS bioreactor system. It consists of a 6 m^3-reactor vessel made of carbon steel, gas recirculation system, a leachate recirculation system and a data acquisition system. The bioreactor vessel was designed as a continuous gas flow packed bed reactor. The most important features of the CSCS bioreactor system are its air-tightness, its capability of either aerobic or anaerobic operation and its ability to completely flood the soil, if required. Also, thermal insulation and supplemental heating enables bioreactor operation during winter season, even when the temperature drops to —15℃.

The bioreactor test has been in progress since September 11. Approximately 4 tons of soil from the IETU site, contaminated with 1L (~1640 g) of TCE and amended with sewage sludge has been bioremediated under anaerobic conditions. Results obtained to date (i.e., decreasing TCE concentration, occurrence of considerable amounts of dichloroethenes (DCEs), and occurrence of VC and ETH in gas phase) indicate that the

stepwise dechlorination of TCE occurs in the bioreactor. Increasing amounts of chloride in the leachate also support this conclusion.

Activities planned for FY02 include:

- laboratory studies on the identification and characterization of the bacterial strains responsible for partial or complete TCE dechlorination and determination of their applicability for bioaugmentation;
- minor changes in the bioreactor system in order to improve its operation;
- bioreactor tests on chlorinated solvent contaminated soil (CSCS) bioremediation under anaerobic/aerobic conditions; and
- CSCS bioremediation process modeling.

1 Introduction

One of the most common environmental problems in the United States and Western Europe is soil and groundwater contamination with chemical solvents classified as Volatile Organic Compounds (VOCs). Chlorinated solvents such as tetrachloroethene (PCE), trichloroethene (TCE), trichloroethane (TCA) and carbon tetrachloride (CT) commonly are used as degreasing agents in manufacturing, maintenance and service facilities all over the world and casually are released into the environment, particularly into soils and groundwater. While large areas of contaminated soil justify dedicated remedial operations, smaller areas could be addressed with *ex situ*, on-site batch remediation.

The successful operation of a petroleum-contaminated soils (PCS) bioreactor during FY00 at the Institute for Ecology of Industrial Areas (IETU) has generated interest in applying bioremediation technology to other contaminants identified by the US Department of Energy (DOE). As a result, the construction and application of a second bioreactor was proposed. The purpose of the second bioreactor is to investigate the applicability of this technology to the remediation of chlorinated solvent contaminated soils.

The project entitled Use of a bioreactor to treat chlorinated solvent-contaminated soils was launched in FY01 as a common initiative of the Institute for Ecology of Industrial Areas (IETU) and Westinghouse Savannah River Company (WSRC), operator of the Savannah River Site (SRS) for the United States Department of Energy (DOE).

2 Background

Chlorinated solvents can be biodegraded when used as a primary growth substrate or via a co-metabolic pathway in the following reactions:

_ reductive dehalogenation (halorespiration) and

_ oxidation reactions:

  _ aerobic oxidation,

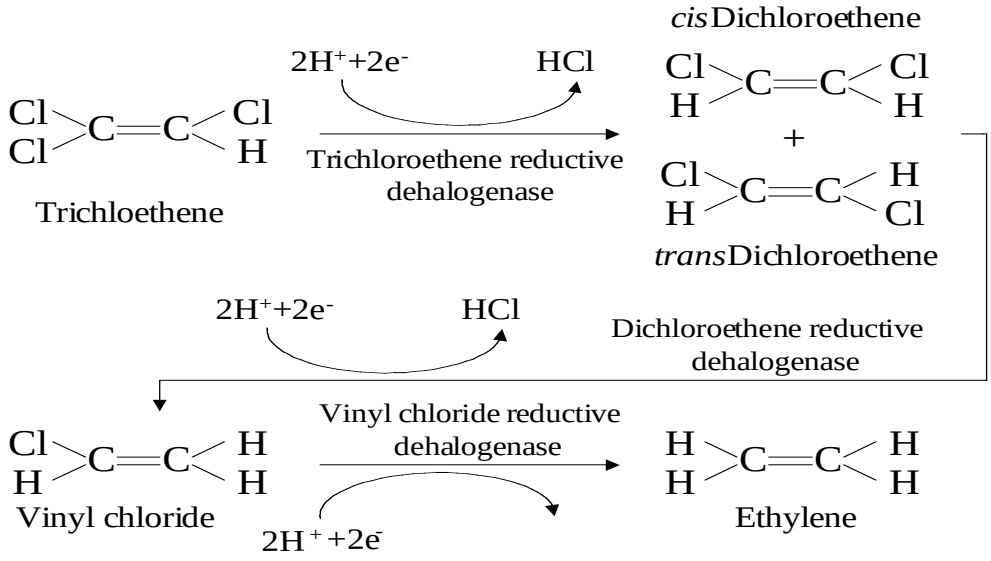
  _ anaerobic oxidation.

It is worth noting that not all chlorinated solvents can be degraded via all of these reactions. Vinyl chloride (VC), for example, is known to degrade via all of these pathways, while trichloroethene (TCE) as a primary substrate can be degraded to dichloroethenes via halorespiration only (Wiedemeier et al. 1999).

There are many reports in the literature on reductive dechlorination of perchloroethene (PCE) and TCE to ethane or ethene by anaerobic mixed cultures of bacteria both in the laboratory and in the field. Many studies also have indicated that selection of an appropriate electron donor may be the most important design parameter for developing

a healthy population of microorganisms capable of dechlorinating PCE and TCE (Smatlak et al., 1996, Fennel et al., 1997, Yang and McCarty, 1998). A typical TCE reductive dechlorination process is presented in Figure 1.

Figure 1 Anaerobic Trichloroethene Graphic Pathway Map



Anaerobic TCE biodegradation pathways are not well understood. It is not clear why some anaerobic systems only partially dechlorinate TCE, while others effect complete dechlorination (Tandol et al., 1994). Results of numerous tests indicate that in the sequential dechlorination of PCE (or TCE) to ETH, VC reduction to ETH is the rate-limiting step. This is supported and reflected in the accumulation of VC, a mutagenic and toxic compound. Nevertheless, if the right microorganisms and conditions are present, complete dechlorination is possible (Loeffler et al., 2000).

It is suggested that in order to avoid cis-dichloroethene (c-DCE) and VC accumulation, this phase of TCE degradation should be carried out under aerobic conditions (Vogel & McCarty, 1985; Bradley & Chapelle, 1996, 1997, 1998; Ferguson & Pietan, 2000). Enzien et al., (1994) showed in column tests that hybrid anaerobic and aerobic biodegradation mechanism combined with methanol as an electron donor resulted in no accumulation of toxic dechlorination products. Aerobic biodegradation of TCE and its daughter compounds is reported by H. Shim et al., (2001). Therefore it was decided that the bioreactor project should allow the process to be carried out under both anaerobic and mixed, anaerobic-aerobic conditions.

3 Project goal and subtasks

The goal of the FY01 study was to develop a technology for bioremediating chlorinated solvent contaminated soil using a small, mobile bioreactor. A packed bed reactor with a treatment capacity of around 4 m^3 of soil per batch was utilized. The system was intended to treat trichloroethylene (TCE) contaminated soils but could be used to treat other contaminated soils as well. The laboratory tests and bioremediation in the

bioreactor were carried out under anaerobic conditions, although, the bioreactor has the ability to operate aerobically or anaerobically in batch mode. The project had the following components:

_ Subtask 1. Treatability study to define optimal parameters for TCE biodegradation;

_ Subtask 2. Bioreactor design and construction;

_ Subtask 3. Bioreactor start-up; and

_ Subtask 4. A bioremediation test treating TCE contaminated soil in the bioreactor.

4 Material and Methods

4.1 Material

Studies were carried out using soil that was contaminated artificially with TCE (ca 200 mg/kg). Soil and sludge samples were characterized/analyzed for certain physico-chemical and microbiological parameters (see tables 2 and 3).

4.2 Analytical procedures

Chemical Analysis

Table 1. Analytical methods

Soil		
Constituent	**Sample preparation and analytical method**	**Instruments**
Particle size distribution	Casagrande's aerometric method in Pr szynski's modification, PN-R-04032:1998	Standard lab equipment
Temperature		Thermocouple IT-BA-7 NiCr-NiAl (K-type) (INTROL S.C., Poland)
pH in H_2O and pH in KCl	According to ISO 10390 Soil quality — Determination of pH	CX731 pH-meter (Elmetron, Poland) with a combined electrode and a temperature compensation probe.
Conductivity	PN-ISO-11265	Same as above
Ignition losses at 600°C	Gravimetric method	Standard lab equipment
Redox potential	According to ISO/DIS 11 271.2: Soil quality — Determination of redox potential — Field method.	Redox electrodes (HYDROMET, Poland)
Organic carbon	Walkley and Blackis method	Standard lab equipment
Available phosphorus (P_2O_5)	Egner-Riehm's method PN-R-04023:1996	CARY 1 VARIAN UV-Vis
Available potassium (K_2O)	Egner-Riehm's method. PN-R-04022:1996	VARIAN Spectr AA-300
Total Kjeldahl Nitrogen	According to ISO 11261	B chi B-426:B-323
Phosphorus	Extraction with 1 M 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 PN-73/C-04576.06 PN-87/C-04576.07 (cadmium column)	CARY 1 VARIAN UV-Vis
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 colorimetric indophenol method	CARY 1 VARIAN UV-Vis

Constituent	Sample preparation and analytical method	Instruments
TPH	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
Gas		
VOC	Gas chromatographic (GC) method	HP 6890 Gas Chromatograph (with Rtx-Volatiles capillary column and a GS-Gastro column) with FID detector
Carbon dioxide		Multi-gas Monitor Type 1302 Br_el@Kj l_
Temperature and moisture		Miniature humidity (and temperature) transmitter with analogue output EE 06 FT 1 A3 E+E ELEKTRONIK, Austria
Leachate		
VOC	Gas chromatographic (GC) method and static head-space technique	HP 6890 Gas Chromatograph (with Rtx-Volatiles capillary column and a GS-Gastro column) with FID detector
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	Same as above
Ammonium	ISO 7150/1:1984 Water quality — Determination of ammonium — Part 1: Manual spectrometric method.	Same as above
Specific conductance	EN 27888: 1993	CX731 conductivity meter (Elmetron, Poland) with a glass cell and temperature compensation probe
pH	PN-90/C-04540.01	Same as above

Microbiological Analysis

The microbial measurements included the following: total number of microscopic fungi on malt extract agar (MEA) with chloramphenicol (100 mg/l) at 25 °C, the number of mesophilic fungi on MEA with chloramphenicol (100 mg/l) at 37 °C, total number of bacteria on standard methods agar (SMA) at the same temperature (i.e., 25 °C and 37°°C). For

these measurements, a dilution technique was employed. Apart from the plating method, total bacterial and fungal numbers were determined with the epifluorescence DAPI (for bacteria) and Calcafluor white (for fungi) methods. Microbial activity was measured with the TTC (Triphenyltetrazolium chloride) method.

The DAPI epifluorescence method provides the total direct number of bacterial cells in soil and leachate, using 4,6-diamindino-2-phenylindole as a fluorochrome (Kepner and Pratt, 1994). The CFW epifluorescence method gives total direct counts of fungal propagules in soil or leachate, using Calcafluor white as a fluorochrome (Sparkes et al., 1994). The TTC method provides the total dehydrogenase activity in soil samples (Alef & Nannipieri, 1995). Oxidation by microbes, like other types of organic oxidation under aerobic conditions, is linked to the electron transport system (ETS) of the cell. The enzymes of the ETS include a number of dehydrogenases, thus dehydrogenase activity can be used as an overall measure of activity in the 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.

The enrichment method provides the Most Probable Number (MPN) of soil or leachate microorganisms (Gerhardt, 1981). All microbiological parameters are calculated per gram of soil dry weight or per mL of leachate.

Microbial identification

Microbial strains were identified by plating on selective microbiological media and presumptively identified microscopically (Zeiss Axioscope, Germany). Identification and characterization of microbiological strains were performed according to standard procedures (Gerhardt, 1981). The following were determined in order to identify the taxonomic positions of the isolates:

_ morphological features (Gram staining);

_ culture features in solid and liquid rich medium;

_ motility and H_2S production;

_ sporulation; and

_ biophysical factors of growth (i.e., pH, temperature, oxygen, and salinity). Physiological tests based on API identification systems were performed. The identification was compared with and referenced in *Bergey s Manual of Determination Bacteriology* (1994).

5 Treatability study to define optimal parameters for TCE biodegradation

Research results indicate many factors, which control the biodegradation of chlorinated solvents in soil (Li et *al. 2000*). The main factors include the following:

_ the presence of indigenous bacterial strains capable of degrading solvents under anaerobic and aerobic conditions;

_ the adaptation of bacterial consortia to specific contaminants;

_ the physical and chemical properties of the contaminants; and

_ soil properties (pH, redox potential, nutrients, etc.).

In preliminary experiments conducted in FY00, two TCE-contaminated sandy soils and a sample of sewage sludge obtained from different wastewater treatment plants, were tested for TCE degradation potential. Biodegradation rates in both the soil and sludge were slow under anaerobic conditions. Therefore, experiments carried out in FY01 had three main goals:

_ to obtain several sewage sludges containing bacterial consortia efficient in TCE dechlorination;

_ to determine the effect of an electron donor on TCE dechlorination efficiency in soil mixed with selected sewage sludge; and

_ to isolate bacterial strains from the most efficient consortia and to determine their effectiveness in the TCE dechlorination process.

5.1 Characterization of biological materials for experimentation

5.1.1 Goal

The objective of this activity was to identify and characterize potential sources of bacterial consortia active in TCE reductive dechlorination.

5.1.2 Materials and Methods

Three samples of sewage sludge as inocula and bioremediated soil were selected for the following research:

1. sewage sludge (S1) from the Katowice Panewniki Wastewater Treatment Plant (excess sludge from extended aeration without primary settling tank, after integrated biological process for P, N, and C removal, lagooned);

2. sewage sludge (S2) from the Siemianowice Slaskie Wastewater Treatment Plant (excess sludge from extended aeration without primary settling tank, after integrated biological processes for P, N, and C removal, stabilized in an open digestion

chamber, dewatered in beltpress);

3. sewage sludge (S3) from the Miechowice Wastewater Treatment Plant (excess sludge from extended aeration without primary settling tank, after integrated biological processes for P.,N, and C removal, dewatered in centrifuges, composted with plant remains); and

4. soil from the IETU s site.

Samples were evaluated for physico-chemical and microbiological properties significant to the bioremediation process. The analysis focused mainly on the content of nutrients, organic carbon and pH. The microbial parameters were as follows: total number of microscopic fungi, total number of mesophilic fungi and total number of bacteria. In these measurements, a dilution technique was employed. Microbial activity was measured with the TTC method.

Methods used in the physico-chemical and microbiological analyses are described in Section 3 of this report.

5.1.3 Results

Results of the analyses are presented in tables 2 and 3.

Table 2. Physico-chemical characteristics of sewage sludge and soil samples

Parameters	Units	Sewage sludge (S1)	Sewage sludge (S2)	Sewage sludge (S3)	Soil
pH (in H_2O)		6.68	6.81	6.9	7.22
pH (in KCl)		6.47	6.42	5.93	6.81
Conductivity	μS/cm	1,498.00	965.70	3,026.00	302.20
$N\text{-}NO_3^-$	mg/kg dry soil	< 0.25	< 0.25	11.5	3.15
$N\text{-}NO_2^-$	mg/kg dry soil	0.88	0.47	12.5	0.31
$N\text{-}NH_4^+$	mg/kg dry soil	12.64	10.53	30.45	0.048
Total N (according to Kjeldahl)	% dry soil	4.76	4.66	1.97	0.21
Total C	% dry soil	29.25	28.96	17.56	2.07
Hydrolytic acidity	meq/kg dry soil	254.50	171.90	87.00	10.60
Exchange acidity	meq/kg dry soil	140.00	148.00	5.5	36.00
K available	mg K_2O/kg dry soil	2,545.00	2,630.00	181.63	238.00
P available	mg P_2O_5/kg dry soil	1,606.00	7,357.00	688.00	253.00
Total P	mg/kg dry soil	1,434.00	10,351.00	6,468.00	5,346.00
Total P (as P_2O_5)	mg/kg dry soil	3,290.00	23,720.00	14,811.00	12,250.00
Total S	% dry soil	0.33	0.32	0.57	0.06
$S\text{-}SO_4^{2-}$	% dry soil	0.21	0.19	0.326	0.014
TPH	mg/kg dry soil	16,224.00	24,683.00	11,205.00	225.00
TPOC	mg/kg dry soil	30,731.00	36,934.00	25,097.00	516.00
Humidity	% dry soil	87.60	85.80	89.4	16.50
Soil classification	Name:	Heavy clay	heavy clay	sandy clay	sandy clay
Size distribution					
Fraction 1 - 0.1 mm	%	39	45	80	60
Fraction 0.1 - 0.05 mm	%	1	2	1	7
Fraction 0.05 - 0.02 mm	%	1	1	1	9
Fraction 0.02 - 0.005 mm	%	0	0	0	8
Fraction 0.002 - 0.002 mm	%	0	1	1	6
Fraction below 0.002 mm	%	59	51	17	10

Table 3. Microbiological parameters of sewage sludge and soil samples

Parameters	Sewage sludge (S1)	Sewage sludge (S2)	Sewage sludge (S3)	Soil
Total number of microscopic fungi/mL	3.5E+05	3.0E+05	4.4E+01	4.5E+04
Total number of mesophilic fungi/mL	3.6E+04	3.5E+04	3.7E+05	2.7E+04
Total number of bacteria/mL	4.4E+07	1.1E+07	4.1E+05	4.1E+06
Total number of mesophilic bacteria/mL	7.0E+05	2.1E+05	2.0E+02	2.1E+05
TTC Microbial activity (mg TPF/g soil)	375	414	397	0.82

Results show the advantageous chemical composition of the analyzed samples. All samples are neutral in pH (pH about 7). Although the soil is not nutrient-rich, the sludges contain large quantities of available K, P and $N\text{-}NH_4^+$. Also, the low content of $N\text{-}NO_3^-$, which undergoes reduction under anaerobic conditions is advantageous.

In all samples, the numbers of fungi and bacteria were high, but only the sewage sludges showed high microbial activity, ranging between 375-414 mg TPF/g sample. Soil microbial activity was relatively low, 0.82 mg TPF/g soil.

High densities of bacteria and fungi are not correlated with dehydrogenase activity (DHA). DHA reflects a broad range of oxidative processes, which are dependent on many parameters such as: the content and composition of organic matter as well as the temperature and humidity of the samples. The parameters charge/change and alter the biological activity of soil microorganisms through the modification of their microenvironments.

The selected sewage sludge served as a good substrate for growing different microorganisms under both aerobic and anaerobic conditions.

5.2 Experiment 1: Determination of TCE dechlorination efficiency under anaerobic conditions for characterized sludges

5.2.1 Goal

The goal of the experiment was to investigate whether the sewage sludge and soil samples chosen contain microbial consortia capable of efficient TCE dechlorination under anaerobic conditions.

5.2.2 Methods

The methods applied were a compilation of those used by Tokunaga *et al.* (1998), Lee *et al.* (1997) and Doong *et al.* (1997). To determine the potential for TCE biodegradation, 7 g of the sludge and soil samples as an inoculum were placed in 125 mL serum bottles filled with 50 mL of minimal broth (MB) each. The MB composition was as follows (per 1L of deionized water) (Table 4):

Table 4. The composition of minimal broth (MB)

Component	Quantity	Purpose
Potassium dihydrogen phosphate	7 grams	Standard compounds for maintenance and growth of 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 headspace of each bottle was flushed with deoxygenated N_2. Then, bottles were sealed with a Teflon-coated rubber and aluminium septum-cap. TCE was added to bottles using a microsyringe to obtain a final concentration of about 117 mg/L. Incubation proceeded at 25 °C for 3 months. Six replicates of each combination of the experiment, (i.e., blank, three sludge samples identified as S1, S2, S3 and soil samples) were prepared. During the test, citrate was used as an electron donor. At the beginning and end of the experiment, concentrations of TCE and its derivatives, and the pH of the medium were determined.

The serum bottles were opened after headspace analysis, and pH and microbiological parameters in the liquid phase were determined. The liquid cultures were used in the experiment designed to isolate TCE dechlorinating microorganisms.

5.2.3 Results

Experiment results are presented in Table 5.

Table 5. VOC content in samples during the experiment

Time, day	µg/sample														
	S1			S2			S3			Soil			Control		
	TCE	c-DCE	VC	TCE	c-DCE	VC	TCE	c-DCE	VC	TCE	c-DCE	VC	TCE	c-DCE	VC
0	5790	4.0	0.0	5790	1.7	0.0	5790	0.0	0.0	5790	0.0	0.0	5790	0.0	0.0
30	2556	2049	0.0	5050	32.5	1.2	4636	0.0	1.3	5723	0.0	0.0	5566	0.0	0.0
60	22	3254	0.0	3620	29.7	2.3	3620	0.0	4.9	4632	0.0	0.0	5027	0.0	0.0

Table 6 TCE content in samples (% of initial content) after the experiment

	S1	S2	S3	Soil	Control
TCE [% of initial content]	0.05	62.6	62.6	80.0	86.8

The results showed the following:

_ in sludge S1, the final TCE content was very low, the DCE content relatively high and no traces of VC were observed; and

_ sludges S2 and S3 show results similar to each other but different from sludge S1. TCE content was high, DCE content was low or equal to zero and small amounts of

VC were detected.

5.2.4 Conclusion

The results show that the final content of TCE and daughter products of its reductive dechlorination varies with the sludge sample. Differences can be attributed to the particular composition of bacterial consortia in each of the sludges. Bacterial populations capable of degrading TCE to DCE prevailed in the S1 sludge. In the S2 and S3 sludges, the bacteria responsible for further degradation were probably more numerous and/or active. In connection with this, a new experiment was planned in which a 50/50 mixture of sludges S1 and S2 was selected as a soil amendment.

Sludge S3 was excluded from further experiments because of its higher content of nitrate.

5.3 Experiment 2: Determination of TCE dechlorination efficiency under anaerobic conditions for the mixture of S1 and S2 with different electron donors

According to the literature, (Gibson & Sewell, 1992) the addition of short-chain organic acids or alcohol will stimulate reductive dechlorination of TCE in anaerobic microcosms. Hydrogen produced during the metabolism of the fatty acids and alcohols may serve as an immediate electron donor for supporting dehalogenation in the system. Numerous microcosms and site studies have shown successful stimulation of dechlorination with substrates such as methanol, ethanol, lactate and butyrate (Fennell & Gossett, 1997).

5.3.1 Goals

The goals of the experiment were:

- to determine TCE dechlorination efficiency under anaerobic conditions for a mixture of two sewage sludge samples; and
- to compare TCE dechlorination results for two electron donors.

5.3.2 Methods

The experiment was designed according to previously used methods, with some modifications: two sewage sludge samples were combined in serum bottles and different electron donors, citrate and methanol, were used. The mixture of sewage sludge samples was 3.5 g S1 + 3.5 g S2, amended with approximately 10 μmol/bottle or ≈ 0.2 mM of methanol or citrate.

5.3.3 Results

Measurements were performed at the beginning of the experiment and then every 10 days until total removal of TCE, was reached. Changes in substrate concentrations and daughter products reductions are presented in Table 7 and Figure 2.

Table 7. VOC content in samples during the experiment

μg/sample									
Duration	**M (with methanol)**			**C (with citrate)**			**Control**		
Days	**TCE**	**c-DCE**	**VC**	**TCE**	**c-DCE**	**VC**	**TCE**	**c-DCE**	**VC**
0	4959.4	1.0	0.0	5025.0	1.0	0.0	5890.0	0.0	0.0
10	4085.8	41.2	0.0	4738.2	64.7	0.0	5714.0	0.0	0.0
20	3730.0	309.5	0.0	3730.3	269.1	0.0	5401.0	0.0	0.0
30	2397.0	1626.2	0.0	3474.0	290.4	0.0	5497.0	0.0	0.0
40	1040.3	1626.2	0.0	3321.7	251.7	0.0	5500.0	0.0	0.0
50	460.4	1962.9	0.0	2031.8	764.7	0.0	5707.5	0.0	0.0
60	128.3	1780.3	0.0	12.6	2323.2	0.0	5621.5	0.0	0.0
70	155.3	1352.3	116.2	0.0	2687.3	0.0	4191.5	0.0	0.0
80	0.0	542.1	271.0	26.2	1781.1	164.6	4259.2	0.0	0.0
90	0.0	195.8	165.4	177.6	648.8	401.8	4428.5	0.0	0.0
100	0.0	119.2	84.5	21.9	232.6	244.9	4395.0	0.0	0.0
110	0.0	50.1	36.2	0.0	50.2	67.2	4378.5	0.0	0.0
μmol/sample									
	M			**C**			**Control**		
Days	**TCE**	**c-DCE**	**VC**	**TCE**	**c-DCE**	**VC**	**TCE**	**c-DCE**	**VC**
0	37.8	0.0	0.0	38.3	0.0	0.0	44.9	0.0	0.0
10	31.1	0.4	0.0	36.1	0.7	0.0	43.6	0.0	0.0
20	28.4	3.2	0.0	28.4	2.8	0.0	41.2	0.0	0.0
30	18.3	16.8	0.0	26.5	3.0	0.0	41.9	0.0	0.0
40	7.9	16.8	0.0	25.3	2.6	0.0	41.9	0.0	0.0
50	3.5	20.3	0.0	15.5	7.9	0.0	43.5	0.0	0.0
60	1.0	18.4	0.0	0.1	24.0	0.0	42.8	0.0	0.0
70	1.2	14.0	1.2	0.0	27.8	0.0	31.9	0.0	0.0
80	0	5.6	2.8	0.2	18.4	1.7	32.5	0.0	0.0
90	0	2.0	1.7	1.4	6.7	4.2	33.8	0.0	0.0
100	0	1.2	0.9	0.2	2.4	2.5	33.5	0.0	0.0
110	0	0.5	0.4	0.0	0.5	0.7	33.4	0.0	0.0

Results obtained after 10 and 20 days provided evidence that the dechlorination process had begun and showed low initial c-DCE concentrations. However, after 30 days, a progressive accumulation of DCE (particularly in samples with methanol) was observed. Measurements after 40 days showed a further increase in DCE content. A drop of 80% in the initial TCE concentration was achieved in samples with methanol; while in citrate samples, only a 50% drop was documented. No detectable VC or ETH concentrations were observed in either variant.

Measurements made after 60 days showed that TCE concentration in both series dropped to nearly 0. c-DCE concentrations remained high. After 70 days in the samples with methanol and after 80 days in the samples with citrate, a reduction of DCE concentration was recorded, along with increased VC concentrations. The increase of VC concentrations was not stoichiometric when compared to the drop in c-DCE,

probably due to further dechlorination of VC to ethylene.

After 110 days, the total degradation of TCE, followed by the degradation of the intermediate products of DCE and VC transformation to ethylene, were obtained in both series.

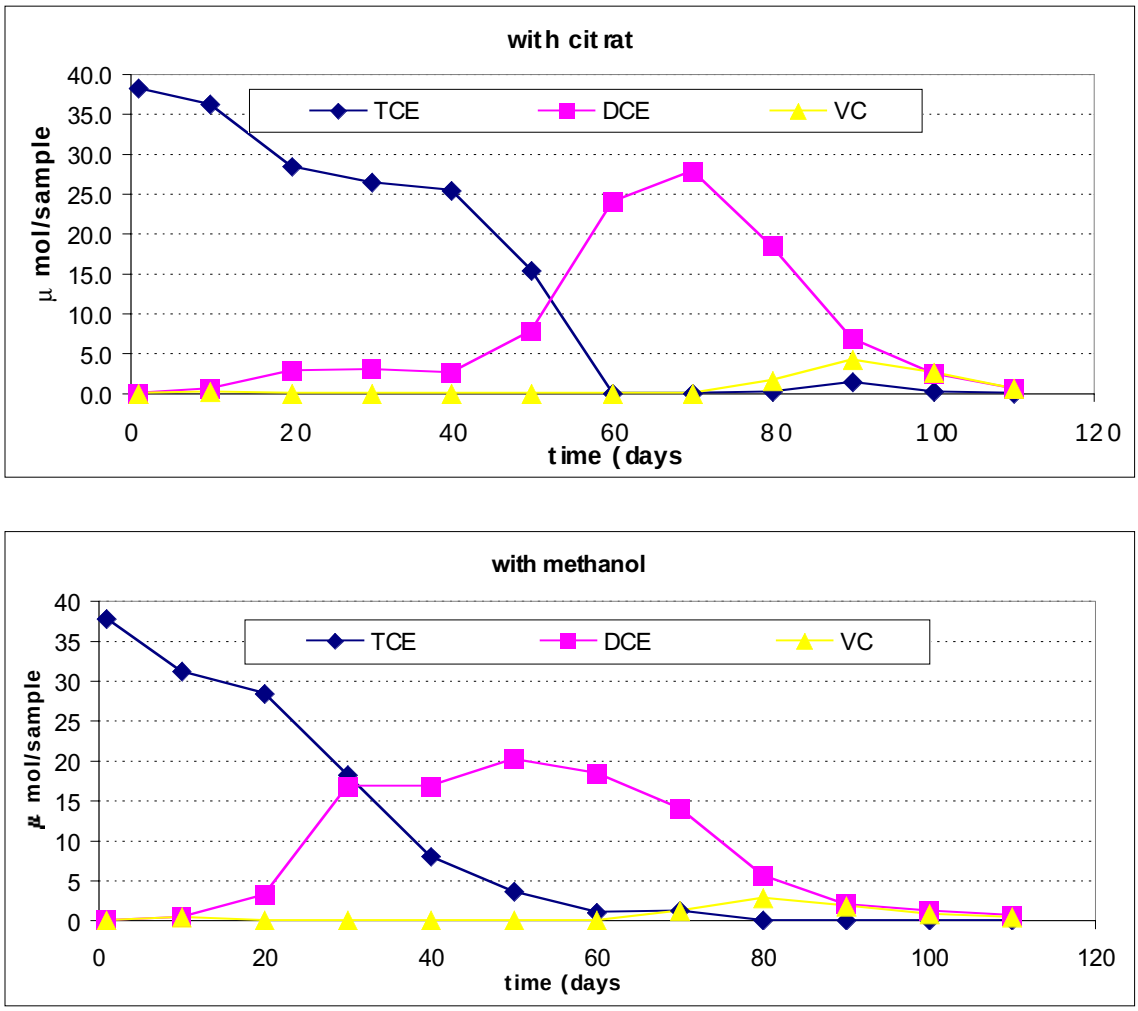


Figure 2. TCE, DCE, VC concentrations vs. time
during bioremediation tests under anaerobic conditions

In anaerobic conditions, sludges used for the experiment generate additional volumes of gas (probably CO_2, CH_4). Peaks in chromatograms, which correspond to ethylene (and ethene) are blurred and thus enable identification of lighter components of the gaseous phase.

Due to difficulties in determining the amount of end product in the dechlorination process the reaction progress was observed via decrement of the sum of substrates and intermediate products (TCE + DCE + VC). No distinct difference in process efficiency was noted between samples with methanol and citrate (Figure˚3).

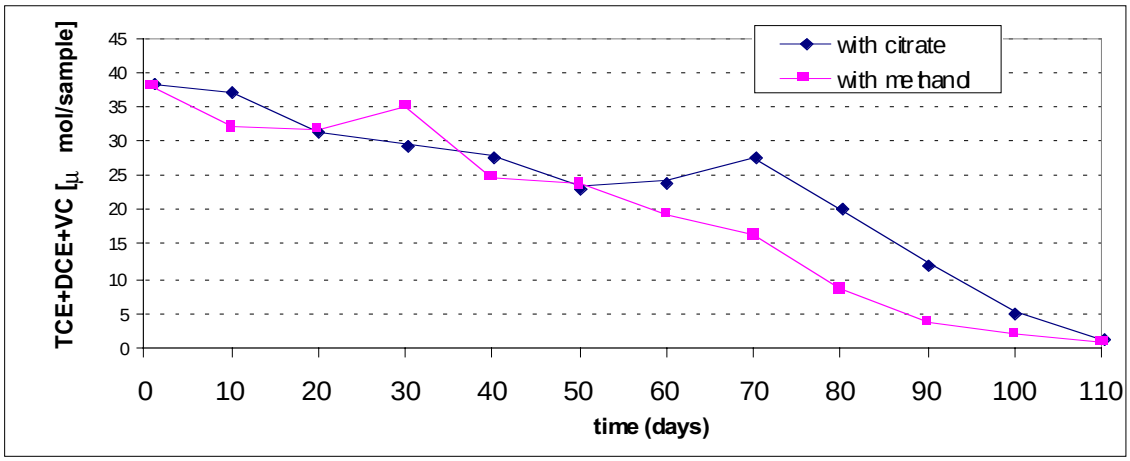


Figure 3. Sum of concentrations of TCE, DCE and VC during the dechlorination process

To determine concentrations of ETH, a GS-Gastro column was installed in the chromatograph. Compared to the Rtx-Volatiles column, a GS-Gastro column enables a more precise analysis of the gaseous phase as it resolves C2 and C3 hydrocarbons and gives a better shape of peaks and reproducibility for light halogenated hydrocarbons.

Analyses of samples from the serum bottles experiment saved at about 4 °C for over three months until the new chromatograph column was operating showed high concentrations of ethylene. This is proof that ethylene is a final product of the anaerobic dehalogenation of TCE in the experiment (see Table 8).

Table 8. Final VOC content in samples saved at 4 °C, 3 months after experiment termination

μg/sample							
M (with methanol)				C (with citrate)			
TCE	DCE	VC	ETH	TCE	DCE	VC	ETH
0.17	7.70	6.16	536	<0.1	6.83	0.27	655

5.3.4 Conclusions

This experiment proved that microorganisms present in the mixture of selected sludges are able to dechlorinate chlorinated ethenes under anaerobic conditions. Changes in substrate and product amounts over time (figures 2 and 3) are in general accordance with the sequential reactions pathway shown in Figure 1. However, in contrast to most literature data, no significant VC accumulation was observed during the experiment. No significant difference between citrate and methanol as hydrogen sources was noted. Methanol, which is less costly, was selected for the remediation test in the bioreactor.

5.4 Experiment 3: Microcosm study

5.4.1 Goals

Goals of this experiment were as follows:

- to evaluate the biodegradation efficiency of TCE and its daughter products in conditions which simulate those expected in the bioreactor;
- to determine the optimal proportions when mixing soil with sludge; and
- to monitor substrate and products concentration in the gaseous phase to determine reaction progress.

5.4.2 Methods

Mixtures of soil and two sewage sludge samples (S1 and S2) in the following proportions were used as microcosms:

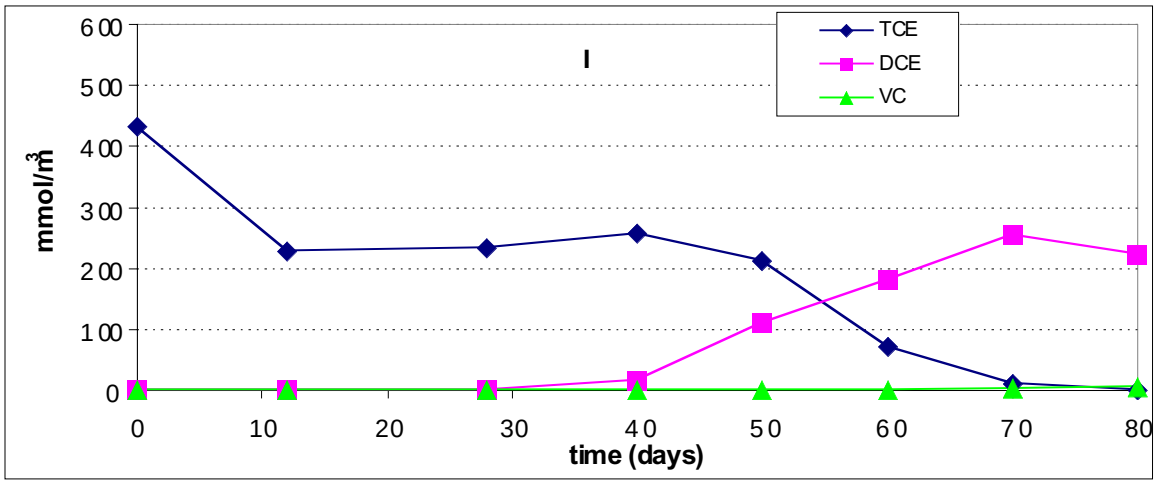
5. soil: S1: S2 = 21:1:1, and
6. soil: S1: S2 = 10.5:1:1.

Each 100 mL serum bottle was filled with 60 g of soil-sludge mixture and 10 μmol/bottle of methanol, then flushed with deoxygenated N_2, and sealed using screw caps containing butyl rubber septa. Appropriate amounts 91 μM of TCE were then introduced into each bottle with a microsyringe.

5.4.3 Results

Reaction progress was observed by monitoring the changes in product and substrates concentrations in the gaseous phase (Figure 4).

After 80 days, a 100 % reduction of TCE was obtained in samples from both series. High DCE concentrations still are detected in both series, however a small amount of VC has been recorded in the second proportion (II). In soil with higher proportion of sludges (series II) the biodegradation of TCE was faster: TCE concentration reached 0% after about 50 days, at the same time small amounts of VC were recorded. In series I 0% concentration of TCE was reached after 70 days and no VC was detected.



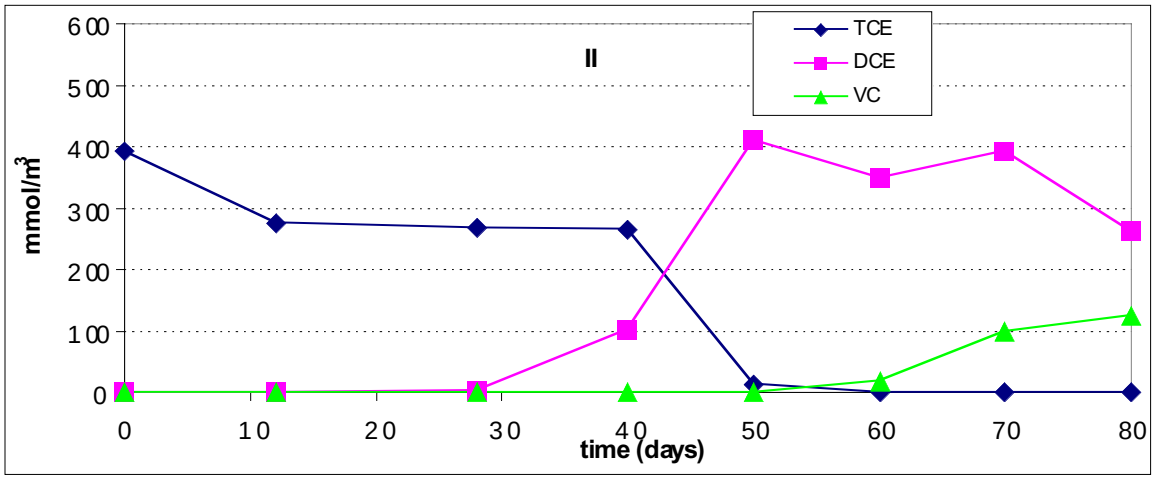


Figure 4 TCE, DCE, VC and ethylene concentrations in gaseous phase vs. time during bioremediation tests under anaerobic conditions
(series I: soil mixed with sludges S1 and S2 in proportion 21:1:1;
series II: soil mixed with sludges S1 and S2 in proportion 10.5:1:1)

5.4.4 Conclusion

The results show the following:

_ the experiment successfully demonstrated TCE bioremediation and enabled for determining conditions of the bioreactor test;

_ measurement of chlorinated solvent concentrations in the gaseous phase allows for monitoring of the dechlorination progress, which means that a similar technique could be employed to monitor bioreactor operation.

5.5 Isolation of bacterial strains

5.5.1 Goals

The purpose of this experiment was to isolate and characterize anaerobic bacterial strains that are able to dechlorinate TCE with the intent to use this knowledge for further studies.

5.5.2 Methods

Bacterial strains were isolated from anaerobic enrichment cultures in sewage sludge samples, labeled S1, S2 and S3, from Experiment 1. The total number of bacterial colonies in anaerobic enrichment cultures of S1, S2 and S3 counted by MPN (Gerhardt, 1981; EPA Standard Method) are presented in Table 9.

Table 9. Total number of bacteria in samples

Sample	S1	S2	S3
Number of bacteria /cm^3	3631	811	991

Cultures were grown under anaerobic conditions at 25 °C for 21 days on minimal broth medium (MB) (composition of the medium, see Table 4) with 20 g/L Bacto Agar (Difco). During incubation, bacteria were exposed continuously to TCE vapors. 20 µl of TCE were put on the Petri dishes. Bacteria were grown in Becton Dickinson anaerobic glass jar. Isolates were obtained by plating serial dilutions on 1/10 SMA medium. Colonies growing on the medium were of various colors (yellow, pink and white) and shapes.

In total, 100 colonies were selected randomly from three replicate plates of each treatment. Isolate purity was confirmed by the sequential plating method.

Finally, 21 purified bacterial strains were isolated and characterized, and used for further experiments. To determine the isolates taxonomic positions and morphological and culture features, Gram staining, temperature, pH, and salinity ranges of growth, motility and production of H_2S and sporulation were performed according to standard procedures (Gerhardt, 1981). Physiological traits based on the API identification systems were tested and results were compared/referenced with *Bergey s Manual of Determination Bacteriology*.

5.5.3 Results

Isolated strains were facultatively anaerobic or microaerophilic. Most of these bacteria belong to the gram-negative aerobic/microaerophilic or facultatively anaerobic rods and cocci, according to *Bergey s Manual of Systematic Bacteriology.* Optimal bacterial growing conditions for pH and temperature are between 6.5 and 7.5 and between 30 and 37 °C (mesotrophic), respectively. Growth will occur, however, in temperatures from 10 to 45°C. Some bacteria can grow in lower temperatures, between 4 and 10°C (see tables 10 and 11). A common feature of most isolated bacteria was gliding motility.

Bacteria from genera *Aeromonas*, *Pseudomonas*, *Chryseomonas* and *Alcaligenus* were identified in the examined samples.

In the genus *Aeromonas* two species were found: *Aero salmonicida* (2 isolates) and *Aero. hydrophila* (5 isolates). Usually they are motile and are producing H_2S. They are

facultative anaerobes. Optimal temperature for their growth is 22 — 28^{o} C. Isolates tested positive for oxidase and negative for urease. These bacteria reduce nitrates, and use glucose, arabinose, mannose and citrate as carbon sources.

In the genus *Pseudomonas*, three species were identified: Ps*. cepacia* (4 isolates) *Ps. fluorescences* (1 isolates) and *Ps. pseudomalei* (1 isolates). *Pseudomonas* species have a strictly respiratory metabolism, with oxygen as the terminal electron acceptor, but in some cases nitrate can be used as an alternate electron acceptor and allow anaerobic metabolism to occur. These species are able to assimilate various substrates (glucose, mannose, mannitol, citrate) as carbon sources. Some differences occur in the case of N-acetylo-glucosamine and arabinose assimilation. None of the *Pseudomonas* isolates have the capability to ferment glucose and to produce urease. All strains are oxidaze-positive. *Pseudomonas cepacia* produce β-galactosidase but not urease.

Table 10. Characteristics of isolated bacteria

No.	Isolates	Culture on the solid Rich medium	Gram reaction	Temperature range 4-6°C*	20°C	37°C	45°C	Motility + Prod. H_2S	Sporulation	pH 9	pH 4
1	II/1	Cream-white slime	NEGATIVE straight rods in chains	+/-	+	+	+	+ **H_2S**	+	+	-
2	II/2	Cream-white slime	NEGATIVE long rods in chains	-	+	+	+	- **H_2S**	+	+	-
3	II/3	Cream-white slime	NEGATIVE long rods in chains	+/-	+	+	+	- **H_2S**	+	+	-
4	II/4	Yellow	NEGATIVE big cocci with different Diameter	+++	+	+	-	-	-	+	-
5	II/4bis	Cream-white slime	NEGATIVE long rods in chains	-	+	+	+	+ **H_2S**	+	+	-
6	II/7	Unpigmented	NEGATIVE slender and thick rods	+/-	+	+	+	- **H_2S**	-	+/-	-
7	II/8	Light pink slime	NEGATIVE big and wide rods	-	+	+	+	+ **H_2S**	+	+	-
8	II/9	Cream-white slime	NEGATIVE straight rods	+/-	+	+	+	+ **H_2S**	+	+	-
9	II/10	Light-yellow slime	NEGATIVE big cocci	+++	+	+	+	- **H_2S**	-	+	-
10	II/11	Pink	NEGATIVE Cocci	+	+	+	+	- **H_2S**	-	+	-
11	II/13	Unpigmented	NEGATIVE big rods often in chains	+++	+	+	+	- **H_2S**	+	+	-
12	II/15	white spread	NEGATIVE single cocci	+	+	+	+/-	- **H_2S**	-	+	-
13	II/16	Yellow	NEGATIVE slender rods	+++	+	+	+	- **H_2S**	-	+	-
14	II/17	Yellow	NEGATIVE single cocci	+	+	+	+/-	+ **H_2S**	-	+	-
15	III/2	Cream-white slime	NEGATIVE slender rods in chains	-	+	+	+	+ **H_2S**	+	+	-
16	III/4	Unpigmented	NEGATIVEE rods, clubbed forms	-	+	+	+	-	-	-	-
17	III/5	Cream-white slime	NEGATIVE straight and big rods, often filaments	+	+	+	+	+	+	+	-
18	III/6	Yellow	NEGATIVE cocci tetrades	+/-	+	+	+	+ **H_2S**	-	+	-
19	III/7	Unpigmented slime	NEGATIVE rods, often filaments	+	+	+	-	+ **H_2S**	+	+	-
20	III/9	Cream-white slime	NEGATIVE rods, usually filaments	+/-	+	+	+	+ **H_2S**	+	+	-
21	III/11	cream-white slime	NEGATIVE rods often filaments	++	+	+	-	+ **H_2S**	+	+	-

* - after 1 week incubation

From the *Chryseomonas luteola* species, 7 isolates were identified. Some of them were motile and produced H_2S, but some were not. They grew at a temperature range between 4 — 45°C. Oxidase and urease tests were negative.

The genus *Alcaligenes* was represented only by *Alcaligenes xylosoxidans*. It is an obligate aerobe. Some subspecies are capable of anaerobic respiration in the presence of nitrate or nitrite. Optimal growth temperature is between 20 — 37°C. Colonies on nutrient agar are non-pigmented. Oxidase and catalase tests are positive. Motility occurs with H_2S production. It is chemo-organotrophic, using a variety of organic acids and amino acids as carbon sources.

Table 11. Characteristics of isolated bacteria (additional parameters)

No.	Isolates	Temperature range					Salinity		
		4-6 °C*	20 °C	37 °C	45 °C	60 °C	0.1%(w/v)	6.5%(w/v)	15%(w/v)
1	II/1	+/-	+	+	+	-	+	+	-
2	II/2	-	+	+	+	-	+	-	-
3	II/3	+/-	+	+	+	-	+	+	-
4	II/4	+++	+	+	-	+	+	-	-
5	II/4bis	-	+	+	+	-	+	+	-
6	II/7	+/-	+	+	+	-	+	-	-
7	II/8	-	+	+	+	-	+	+	-
8	II/9	+/-	+	+	+	-	+	+	-
9	II/10	+++	+	+	+	-	+	-	-
10	II/11	+	+	+	+	+/-	+	+/-	-
11	II/13	+++	+	+	+	-	+	+/-	-
12	II/15	+	+	+	+/-	-	+	+	-
13	II/16	+++	+	+	+	-	+/-	-	-
14	II/17	+	+	+	+/-	+/-	+/-	-	-
15	III/2	-	+	+	+	-	+	+	-
16	III/4	-	+	+	+	-	+/-	-	-
17	III/5	+	+	+	+	+	+	-	-
18	III/6	+/-	+	+	+	-	+	+	-
19	III/7	+	+	+	-	-	+	-	-
20	III/9	+/-	+	+	+	-	+	-	-
21	III/11	++	+	+	-	-	+	-	-
* - after one week incubation									

5.5.4 Conclusions

_ Sewage sludge samples are rich in different microorganisms — bacteria and fungi, and are useful for this kind of experiment.

_ Sewage sludge mixtures contain anaerobic bacterial strains taking part in dechlorination of TCE.

_ Isolated bacterial strains were facultatively anaerobic or microaerophilic. They are

mainly from the *Aeromonas*, *Pseudomonas*, *Chryseomonas* and *Alcaligenes* genera. The identification of bacterial strains should be tested further with more sophisticated methods.

_ To confirm the TCE biodegradation by pure bacterial strains, experiments should be continued.

_ Isolated bacterial strains may be responsible for the entire TCE dechlorination pathway, or at least one step.

_ Isolated bacterial strains should be tested for their ability to reductively dehalogenate TCE (Tandol et al., 1994; Harkness et al., 1999).

5.6 Current research

5.6.1 Study of TCE biodegradation by pure bacterial cultures

The following bacterial strains were selected for the TCE biodegradation experiment: *Aeromonas salmonicida* (II/1), *Chryseomonas luteola* (II/2 and III/6), *Pseudomonas cepacia* (II/3), *Pseudomonas fluorescens* (II/4bis), *Aeromonas hydrophila* (II/7), *Alcaligenes xylosoxidans* (III/2), and *Pseudomonas pseudomallei* (III/5). The experiment was conducted in 125 mL serum bottles under anaerobic conditions. 200 μL of bacterial suspension were added as inoculum to 75 mL of minimal broth (MB) (see Table 4).

Growth conditions have been described previously (see section 2.2.2).

TCE and its daughter products (*cis*-1,2-DCE, *trans*-1,2-DCE, 1,1-DCE, VC and ETH) concentrations were measured one and two weeks after the experiment began. TCE and its daughter products were not detected.

Two weeks after the experiment began, cultures were supplemented with methanol (approximately 10 μmol/bottle or ≈ 0.2 mM). One week after the addition of methanol TCE and its daughter product concentrations were measured. Trace concentrations (from 0.25 μg/sample to 1 μg/sample) of intermediates (*cis*-1,2-DCE, *trans*-1,2-DCE and 1,1-DCE) were detected. The experiment is in progress. Depending on results obtained, further experimental conditions will be set.

5.7 Summary of treatability study

Data from the treatability study provided information, which may serve as a basis for determining the physico-chemical and microbiological parameters for the remediation of TCE -contaminated soil in a bioreactor. It was found that bacteria present in the sludge mixture are capable of complete dechlorination of TCE to ethylene in anaerobic conditions. Also, no accumulation of vinyl chloride in the product mixture was observed.

The goal of continued studies is to identify bacterial strains responsible for partial or complete TCE dechlorination and to determine their applicability for bioaugmentation.

6 Bioreactor design and construction

The goal of this subtask was to design and construct a portable, efficient and readily deployable system for treating small quantities of soils contaminated with chlorinated solvents incorporating the experience gained during the design, construction and operation of the PCS Bioreactor.

6.1 Bioreactor design

Generally, the chlorinated solvents contaminated soil bioreactor (CSCS bioreactor) is similar to the PCS Bioreactor constructed in FY00 for bioremediation of petroleum contaminated soils. The most important features of the CSCS bioreactor system are its air-tightness; its capability of either aerobic or anaerobic operation; and its ability to completely flood the soil, if required to create anaerobic conditions in contaminated soil.

The CSCS bioreactor was designed by IETU, in cooperation with SRTC, and constructed by a small Polish firm, Mechanika Maszyn. Mechanika Pojazdowa i Slusarstwo, located in Mikolow, near Katowice. The bioreactor consists of a reactor vessel, a gas recirculation system, a leachate recirculation system (Figure˚7) and a monitoring system (Figure 8).

The bioreactor vessel (Figure 5) was designed as a continuous gas flow packed bed reactor. Instead of modifying a regular waste container (i.e., skidpan), as was done in the case of the PCS Bioreactor, a new 6˚m^3 air-tight vessel was constructed. Experience gained during construction of the PCS bioreactor revealed that incorporating the airtight welding requirements during initial construction of the container was more cost effective than modifying and existing container. A false floor was mounted inside the reactor to support the soil bed and connections were made for gas recirculation, leachate recirculation and data monitoring.

Figure 5. CSCS Bioreactor

In order to prevent the bioreactor soil bed from freezing during winter, a light and easy to remove enclosure made of wood and 15 cm thick pressed polystyrene foam sheets was built around the bioreactor. Inside the enclosure two heaters, 2 kW each were installed for heating the reactor if required (Figure 6).

Figure 6. Insulated bioreactor enclosure.

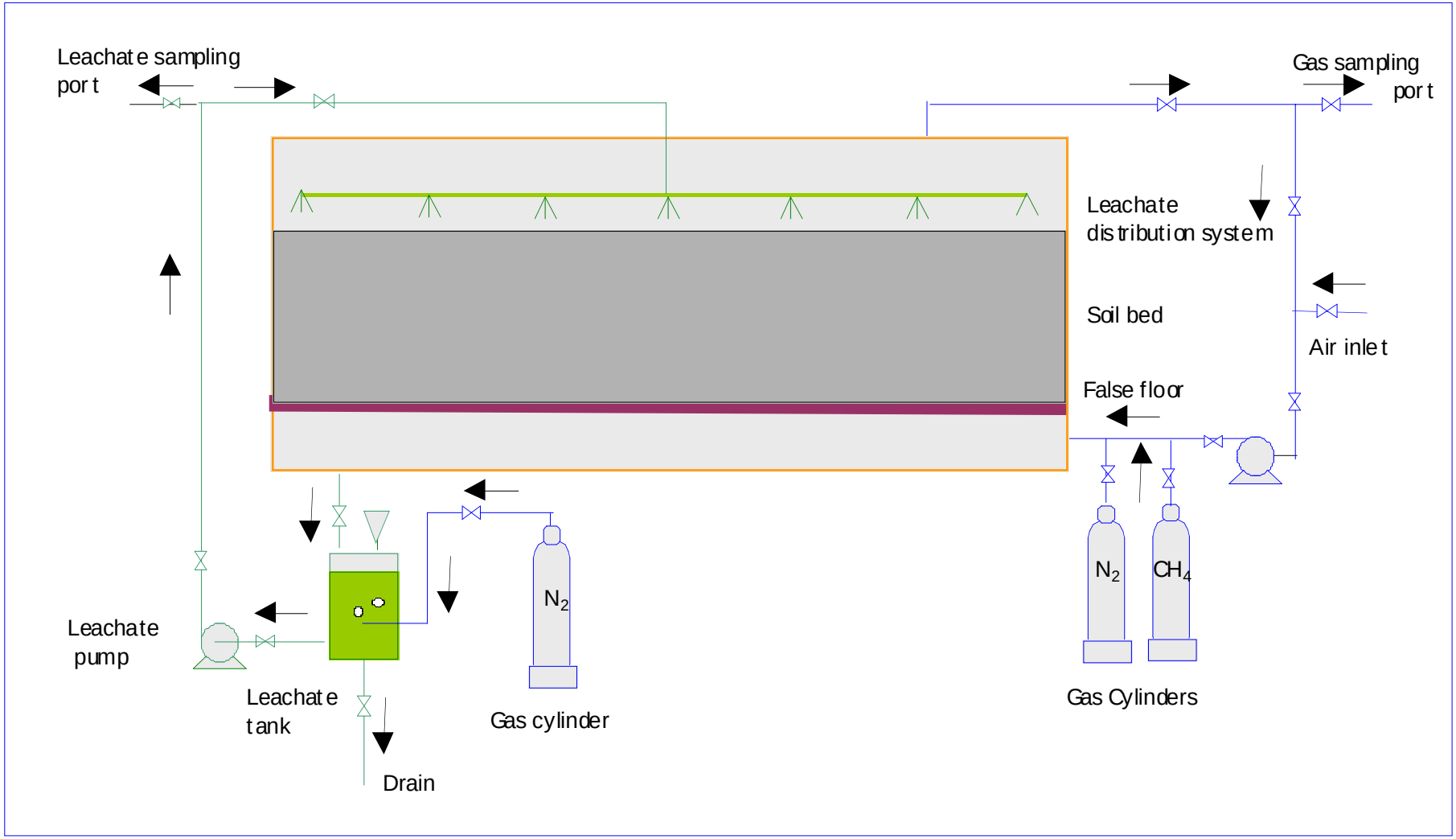


Figure 7. Bioreactor gas and leachate recirculation systems

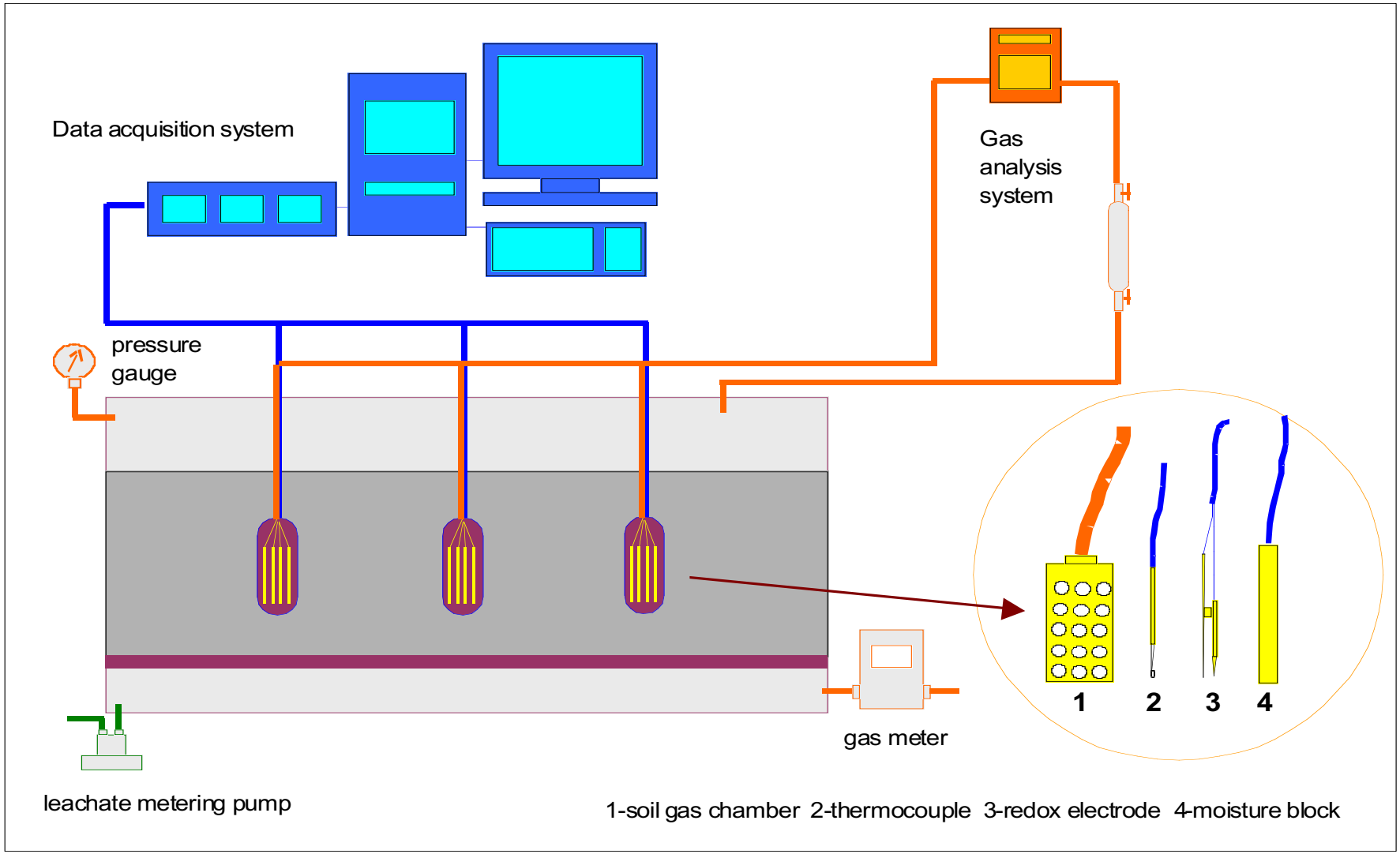


Figure 8. Data monitoring system

The leachate recirculation system consists of a tank, a pump, valves, perforated hoses and process piping. This system has one influent and one effluent connection to the reactor vessel. The effluent process piping, equipped with a shutoff valve, is connected to a tank vessel located beneath the bottom of the bioreactor vessel. Nitrogen from a gas cylinder can be blown through the tank vessel to prevent ambient airflow into the system, if necessary. Leachate from the tank is pumped through a system of parallel, perforated hoses located at the inlet to the reactor, allowing the leachate to be spread uniformly on the upper surface of the soil bed (figures 9, 10 and 11) to control soil moisture and allow the addition of nutrients, etc. as needed.

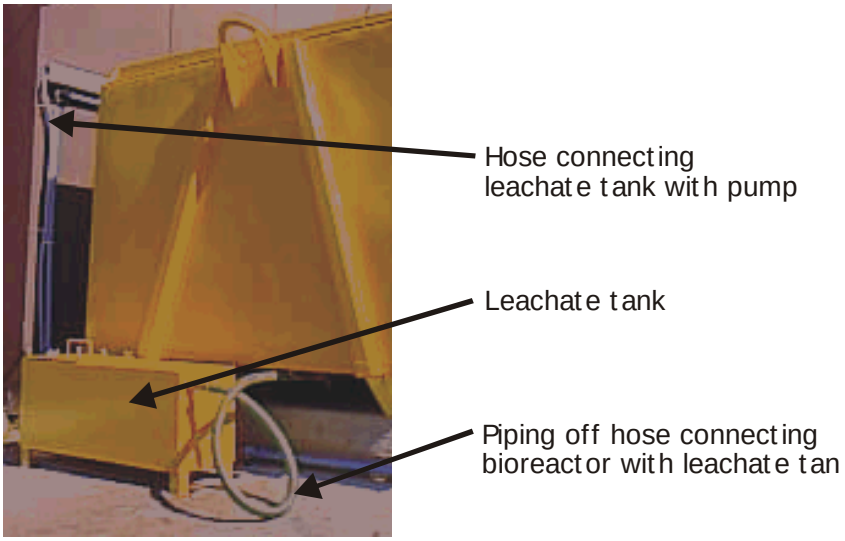


Figure 9. Leachate recirculation system (fragment 2)

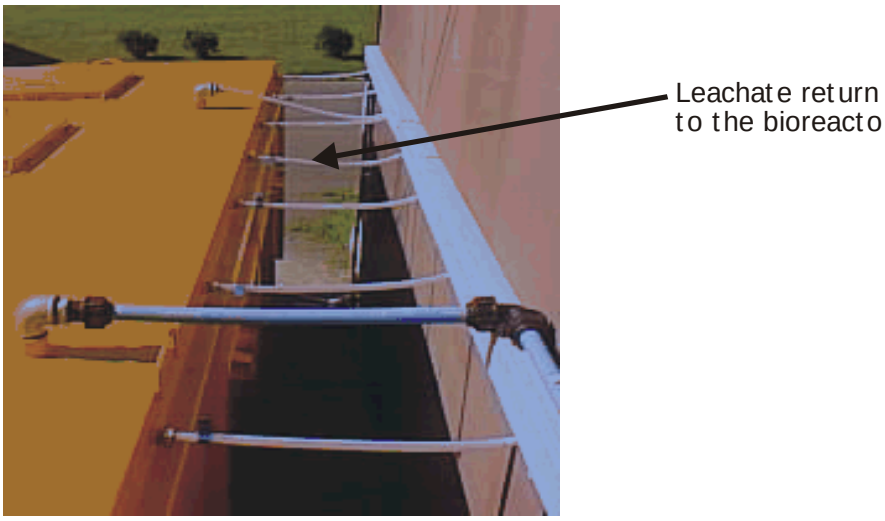


Figure 10. Leachate recirculation system (fragment 3)

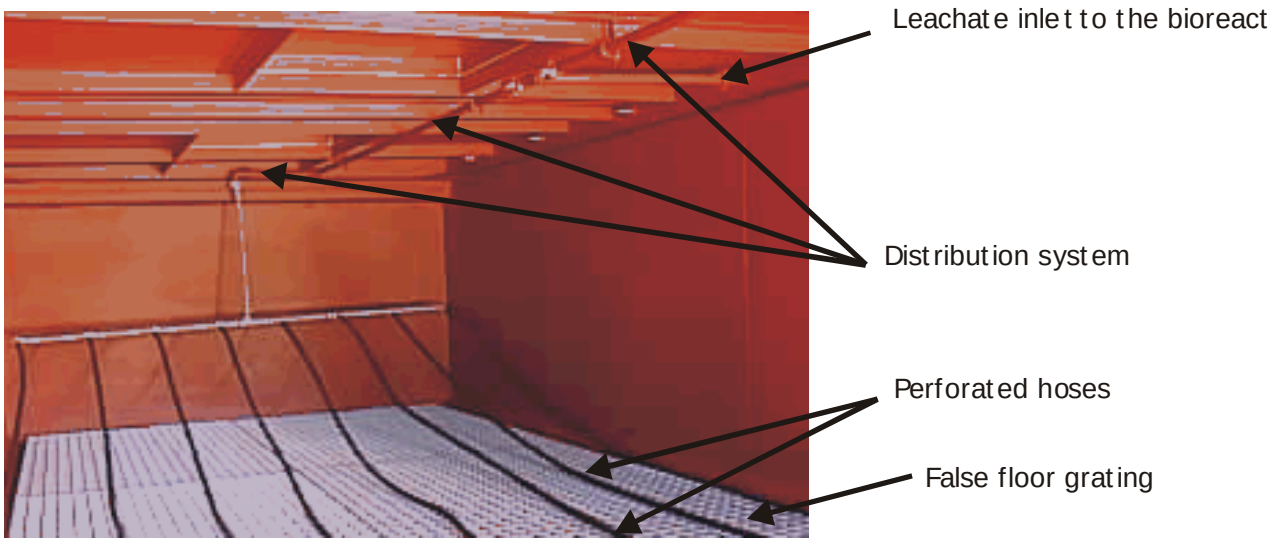


Figure 11. Leachate recirculation system (fragment 4).

The gas recirculation system consists of a blower equipped with an inlet air filter, a bypass pipe equipped with a control valve and an inlet air nozzle. The bypass pipe allows the introduction of inert gas (e.g., nitrogen) or substrate gas (e.g., methane) into the bioreactor (figures 12, 13 and 14).

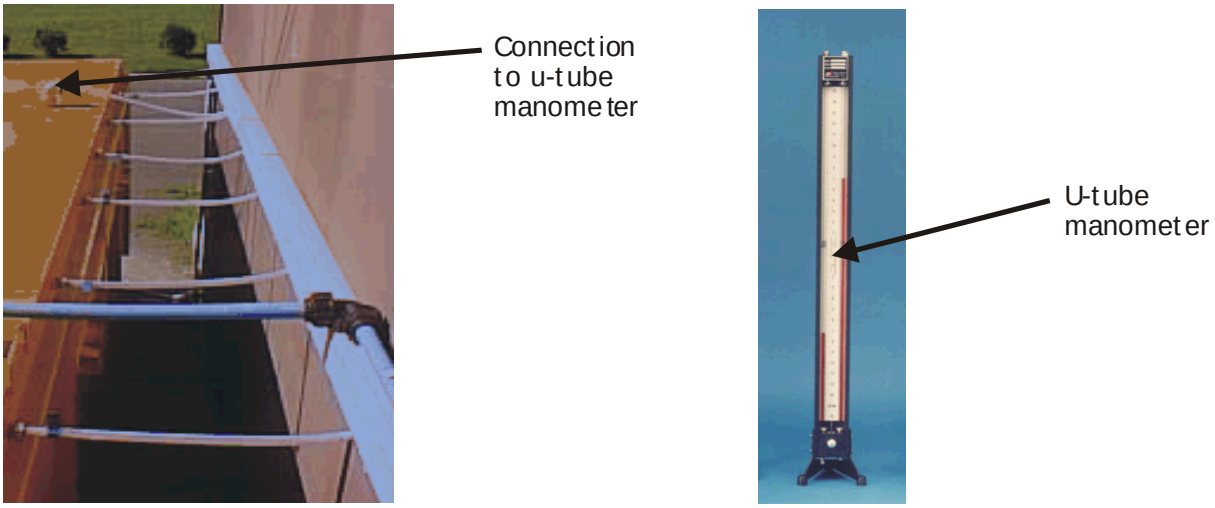


Figure 12. Gas circulation system (fragment 1)

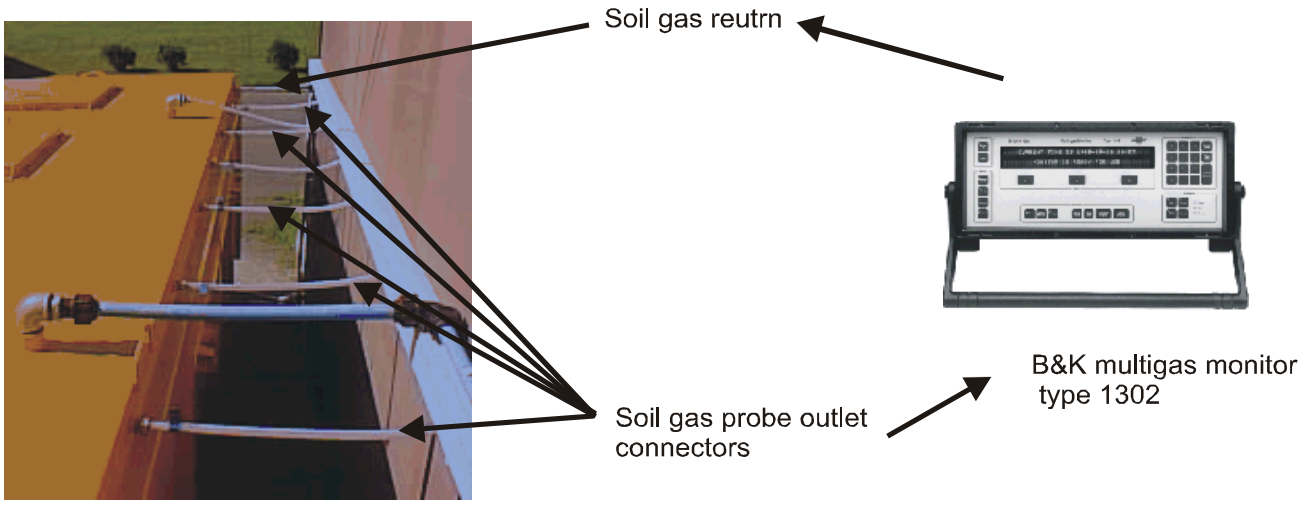


Figure 13. Soil gas-sampling system (fragment 2)

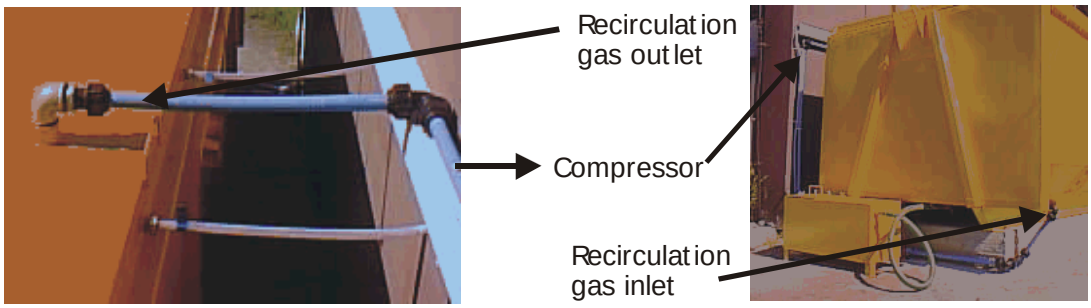


Figure 14. Gas recirculation system (fragment 3)

The reactor system is equipped with sensors for monitoring the main parameters of the bioremediation process.

The monitoring system is designed to collect data, support the control of the bioreactor working parameters and evaluate the bioremediation rate. As reductive conditions have to be maintained in the reactor, there is no opportunity for taking soil samples from the reactor when the bioremediation test is in progress. Information on how bioremediation proceeds has to be obtained from changes in the composition of leachate and soil gases.

The gas sampling system consists of 5 probes distributed evenly along the main axis of the reactor vessel, about 30 cm below the surface of the soil bed. Each probe has a separate connection to the point where soil gas samples are taken using a B&K Multi-gas Monitoring device.

Leachate samples are taken from the leachate tank.

Soil redox potential is measured using five platinum electrodes, each one coupled with one of two silver-silver chloride reference electrodes in such a way that the distance between the redox and the reference electrodes is less than 1 m as required by the electrodes producer. Each redox electrode is located near one of the soil gas probes.

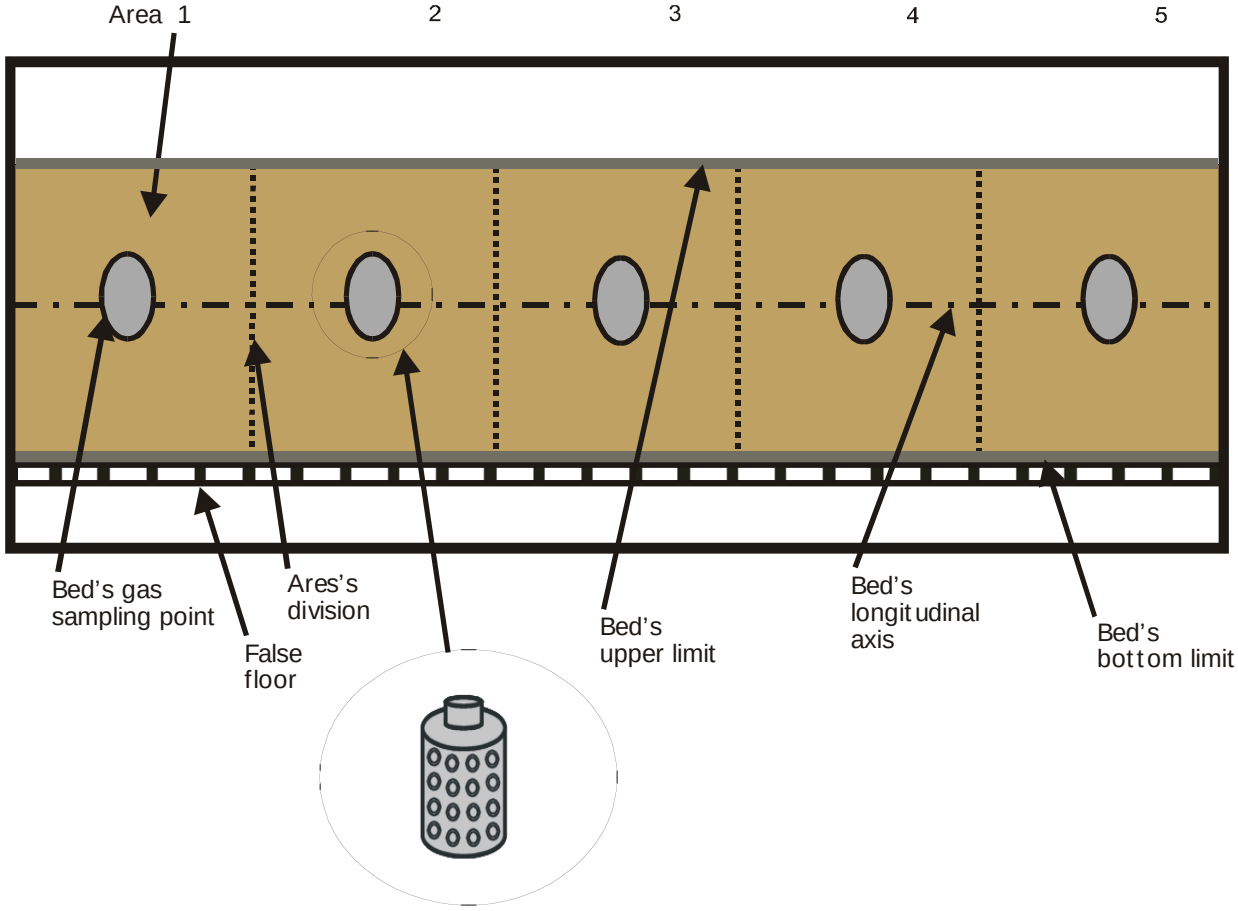
Before installation, the electrodes were calibrated using standard solutions. Checking calibration will be performed after the CSCS bioreactor test is completed.

Temperature is measured at three locations in the soil bed and at one point above the soil, in the vicinity of the gas outlet to the circulation system.

Humidity of the gas phase is measured at the same point as gas temperature.

Parameters measured and sampling frequencies are listed in Table 12.

Figure 15. Gas sampling points in the bioreactor distribution scheme — side view



The data acquisition system was build using Advantech Inc. ADAM 4000 series modules and a PC computer running MS Windows NT 4.0 workstation. Output signals from the sensors are wired to ADAM-4017 or ADAM-4018 modules. These modules include 8 channels 16 bits analog to digital converters. Digital pulses from gas flow meters are wired to ADAM-4080 module, which includes two channels 16 bit counters. This module also is used to control, via power relay module, power supply of the sensors and constant current source module. Experience gained during the operation of the PCS bioreactor determined that constant power to the sensors was not required. Therefore the system automatically switches the sensors on for a few minutes before taking measurements. This solution decreases power consumption and increases the lifetime of the sensors. All modules are connected together via RS-485 interface and to the

computer via ADAM-4522 converter and RS-232 interface. The data acquisition process is controlled fully by IETU created software (called BioReDaq) working in Advantech VisiDaq environment. Using this computer program it is possible to observe direct output sensors signals, measured values expressed in natural units (e.g., % of volumes, etc.) and input parameters (individually for each sensor) used for conversion from the output signal to natural units. The use of BioReDaq also allows scheduling of the 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.

Data acquisition and processing software formatting
Built in FY00 for the PCS Bioreactor the data acquisition system software had to be redesigned in order to meet new requirements of the CSCS Bioreactor aerobic/anaerobic operation. Since the TCE bioremediation project requires automated monitoring, the software was enhanced so that data acquisition from the second bioreactor could be performed from the same application. The software modifications included:

_ enhancing capabilities of measurements averaging during the measurement process,

_ adaptation to new sensor configuration,

_ new user interface,

_ new data logging procedures, and

_ module for TCE bioreactor data acquisition.

The software was developed under the Advantech VisiDaq environment. The MS Access database for measurement, data storage and processing was designed and implemented. Data stored in the database can thus be exported to MSExcel or other software for further analysis or presentation. One computer can be used to control and acquire data from both PCS and CSCS bioreactor systems.

6.2 Bioreactor startup testing

After construction of the bioreactor, startup testing was conducted. This included leak testing of the entire system, water testing of the leachate recirculation system and testing of the data acquisition system. Leak testing of the system included water and gas tests and showed no leaks in the reactor vessel. Water testing was used to check reactor system integrity; the gas testing was done to identify leaks. Testing of the leachate recirculation was carried out in order to identify any possible leaks from the leachate recirculating pump and piping and to establish uniform distribution of the leachate on the upper surface of the soil bed.

7 Experiments in the bioreactor

7.1 Goals

The goals of the experiment initiated on September 10, 2001 are as follows:

_ to evaluate the effects of scaling up the chlorinated solvents degradation process from laboratory scale to bioreactor scale, and

_ to test the data acquisition system.

7.2 Start-up of the bioreactor

About 4 Mg of soil, collected near the IETU, was sieved, mixed with wood chips and sludge S1 and S2 (in the proportion 10.5:1:1) and delivered to the bioreactor (Figure˚16).

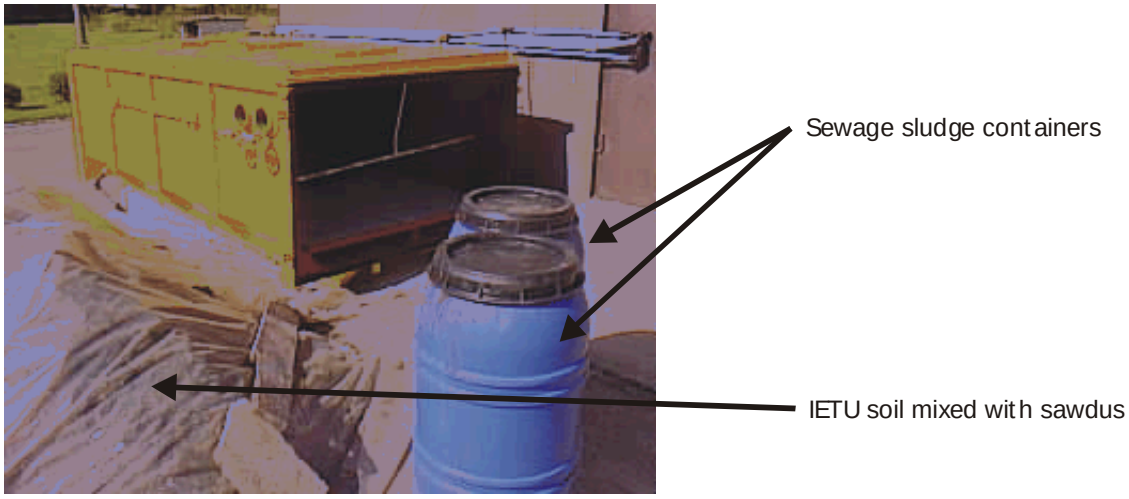


Figure 16. Bioreactor before filling up

The bioreactor was closed tightly and the inside air was replaced by nitrogen to obtain a reductive atmosphere. Then 1 L of TCE (about 400 mg/kg) and 3 L of methanol were mixed with 20 L of water and applied to the bioreactor using the leachate circulation system. Finally, the leachate circulation system was washed with 10 L of water.

After a 3-day stabilization period, gas recirculation and the registration of measurement parameters were initiated.

7.3 Sampling and data monitoring

The following parameters were measured:

_ redox potential, temperature, soil gas composition in the soil bed;

_ concentrations of oxygen, TCE, DCE, VC and ETH, humidity, temperature and pressure in the gas above the soil bed; and

_ pH, TCE, DCE, VC, TPH, nutrients, chloride in leachate.

Samples were taken according to the schedule shown in Table 12 and analyzed at the IETU laboratory using methods listed in Table 1.

Table 12. Bioreactor Data & Sample Plan

Required Parameters	Soil			Gas			Leachate			Analysis
	B	D	F	B	D	F	B	M	F	Type
DAPI and CFW	Yes		Yes							Lab
TPH	Yes		Yes							Lab
TCE	Yes		Yes	Yes	T/W	Yes		Yes		Field/Lab
DCE	Yes		Yes	Yes	T/W	Yes		Yes		Field/Lab
VC	Yes		Yes	Yes	T/W	Yes		Yes		Field/Lab
Ethylene				Yes	T/W	Yes				Field/Lab
Ethane				Yes	T/W	Yes				Field/Lab
% CO_2				Yes	T/W	Yes				Field
% O_2				Yes	Yes	Yes				Field
Redox	Yes	Yes	Yes					Yes		Field
Moisture	Yes		Yes		Yes					Field
NO_2 + NO_3	Yes		Yes					Yes		Lab
NH_4	Yes		Yes					Yes		Lab
TKN	Yes		Yes					Yes		Lab
pH	Yes		Yes					Yes		Lab
Electrical Conductivity								Yes		Lab
PO_4	Yes		Yes					Yes		Lab
Total P	Yes		Yes					Yes		Lab
BOD								Yes		Lab
COD								Yes		Lab
Temperature		Yes			Yes					Field

B - base, D — daily, M — monthly, F — final, T/W — twice a week

Soil sampling protocols: Due to the high volatility of contaminants and their decomposition products, no soil samples were collected from the bioreactor during the bioremediation process. Both the soil and the sludge were analyzed before the process and further analysis will be performed after finishing the soil clean-up process. Soils samples will be collected using a hand auger and will be placed in a Whirl-Pak bag, or other clean container, and transported to the laboratory for immediate analysis according to the plan.

Soil-gas sampling protocols: Gas samples for TCE, DCE, VC, ethylene and ethane analyses are taken from the gas space above the contaminated soil and at the soil sensor locations using a B&K multi-gas monitor. The gas samplers are flushed several times with the gas before the sample is taken and delivered to the IETU laboratory. Gas that flushes the gas samplers is circulated back into the bioreactor.

Leachate sampling protocols: A sampling port is used to collect leachate samples from the leachate recirculation system.

7.4 Bioreactor operation

7.4.1 Leachate recirculation

Leachate is circulated daily for about 8 hours with a flow rate of about 10 L/hr. Due to low amounts of leachate in the bioreactor, 50 L of water were added to the system on September 25.

7.4.2 Gas recirculation

The soil gas is circulated through the bioreactor in closed circuit for 8 hours a day with a flow rate of about 1 m^3/hr.

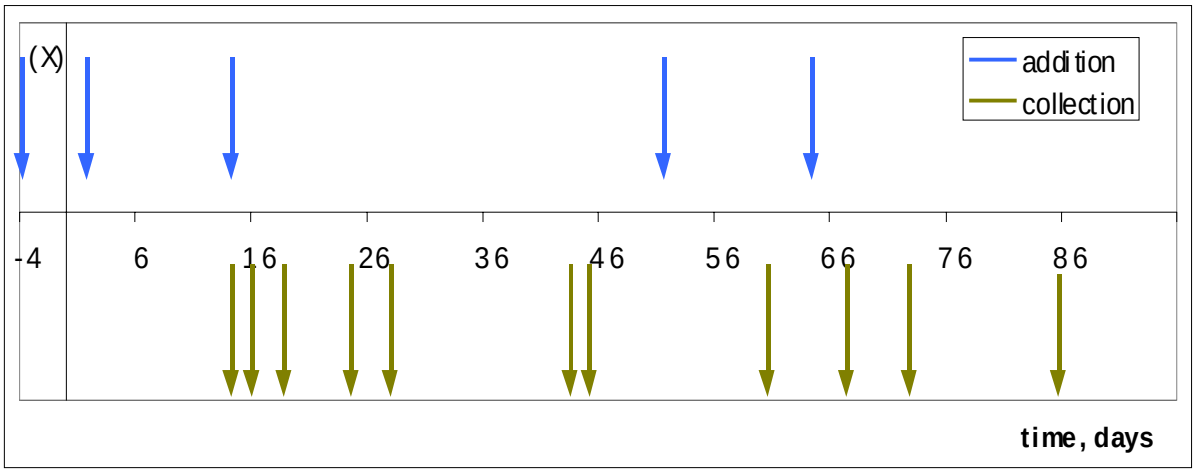
7.4.3 Pressure control

Bioreactor pressure is controlled through a U-tube manometer fixed to one of the gas outlets. Pressure measurement data are recorded daily on every workday. Gas cylinder nitrogen is attached to the gas circulation system and the bioreactor can be flushed, if necessary.

7.4.4 Sampling

The sampling cycle was adjusted to reflect intensity in changes in sample composition. During the first week after contamination, air samples were collected daily but, because their composition did not differ considerably, a biweekly air sampling cycle was established.

Dates of leachate make up and sampling are presented in Figure 17.



(X) - four days before the measurements started

date	day	addition	amount
06.09	-4	TCE	1 L
		methanol	3 L
		water	20 L
12.09	2	water	10 L
25.09	15	water	50 L
29.10	49	methanol	3 L
		water	3 L

date	day	collection	amount
25.09	15	leachate	0.1 L
26.09	16	leachate	1 L
28.09	28	leachate	0.9 L
05.10	25	leachate	1 L
08.10	28	leachate	0.5 L
24.10	44	leachate	0.5 L
25.10	45	leachate	0.9 L
09.11	60	leachate	0.5 L
16.11	67	leachate	0.4 L
21.11	72	leachate	0.5 L

Figure 17. Leachate make up and sampling days.

7.5 Monitoring of the chlorinated solvent contaminated soil bioremediation in the bioreactor - results

7.5.1 Physico-chemical parameters

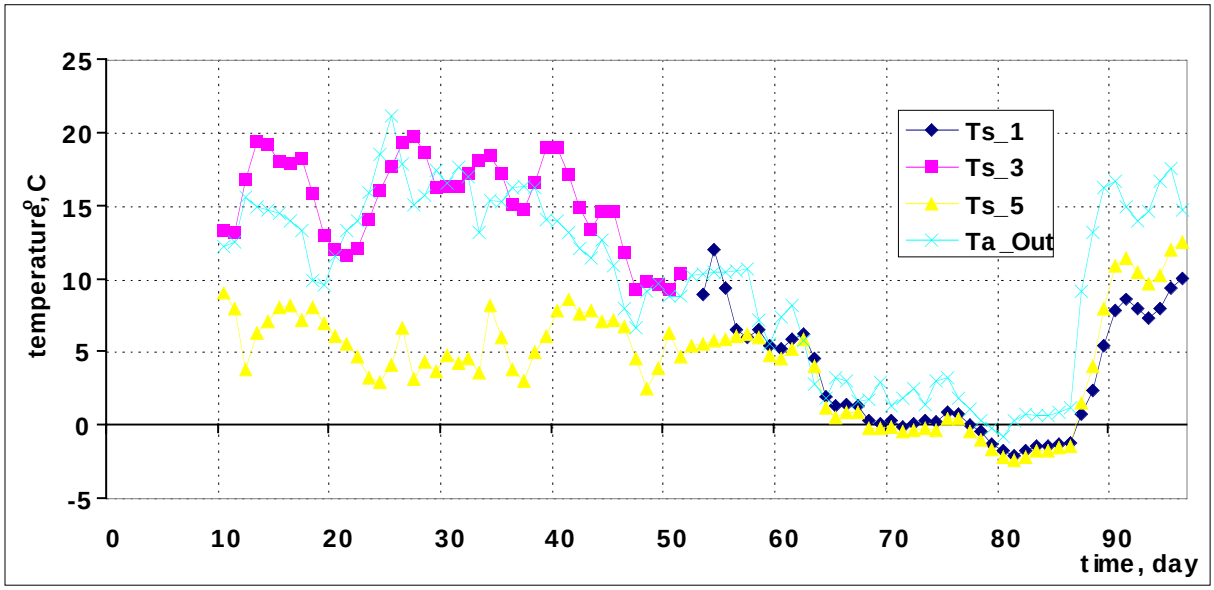
Soil redox potential, soil temperature as well as gas temperature and humidity were measured continuously since day 8 of the process.

Temperature

Mean daily temperatures in the bioreactor (Ts1, Ts3 and Ts5 in soil, Ta out) are presented in Figure 18. As the ambient air temperature decreases, the temperature inside the bioreactor decreases also. As outside air temperature decreases to below 0°Celsius, the temperature inside the bioreactor also decreases to below 0°Celsius. After providing the bioreactor with appropriate thermal insulation (on the 76th day of the

process) and additional heating the temperature increased to above 0°Celsius inside the bioreactor.

Figure 18. Soil and gas temperatures in bioreactor



Pressure

During the entire process pressure inside the bioreactor was slightly above atmospheric pressure (Figure19).

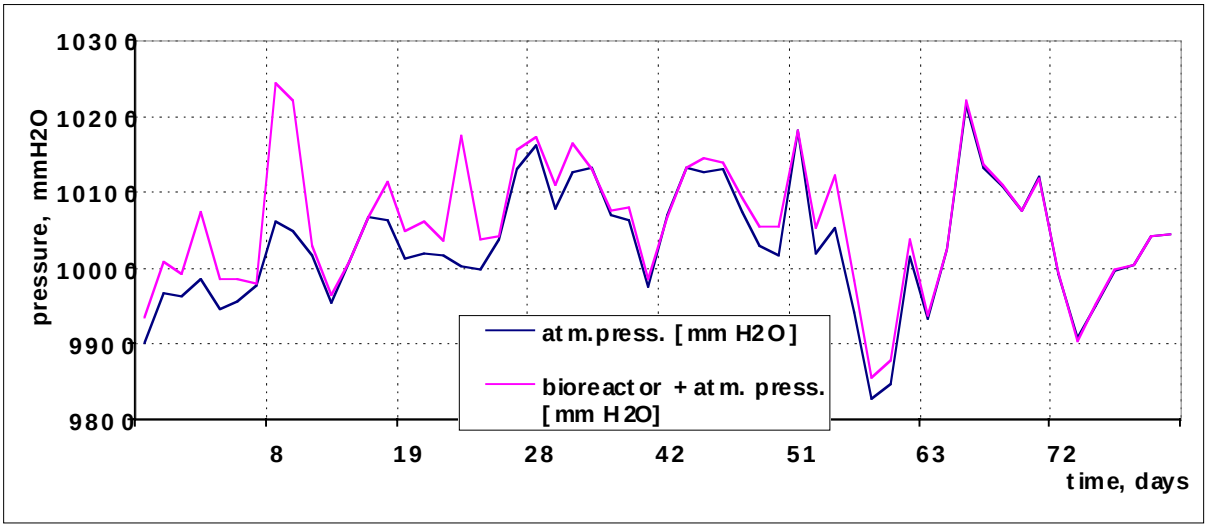


Figure 19. Bioreactor and atmospheric pressure

Humidity

Figure 20 shows data of gas humidity at the gas recirculation system outlet. Measurement results seem to be underrated; however, the presence of leachate indicates that soil in the bioreactor is saturated with water and prolonged circulation of the gas through the soil bed should result in near-complete gas saturation.

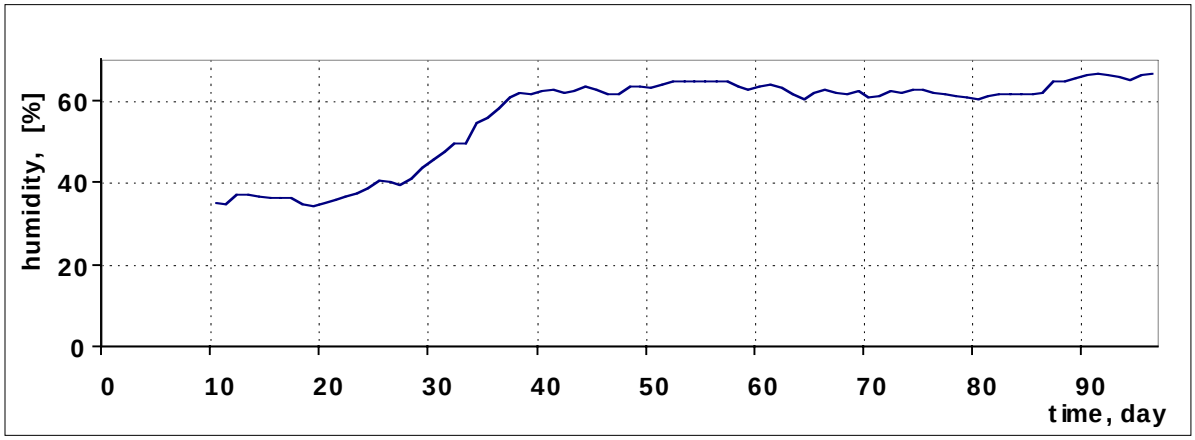


Figure 20. Gas humidity inside the bioreactor

Redox potential

Figure 21 shows changes of redox potential in reference to a silver-silver chloride electrode. Generally, electrodes E1, E2 and E3 (connected to one of the two reference electrodes) show a systematic increase of soil redox potential. In the case of electrodes E4 and E5 (connected to the other reference electrode), one shows a systematic decrease whereas the second one shows a slight increase in soil redox potential. When the temperature inside the bioreactor dropped below 0˚C, all five electrodes indicated a clear increase in redox potential. Thermal insulation and heating of the bioreactor reversed this situation.

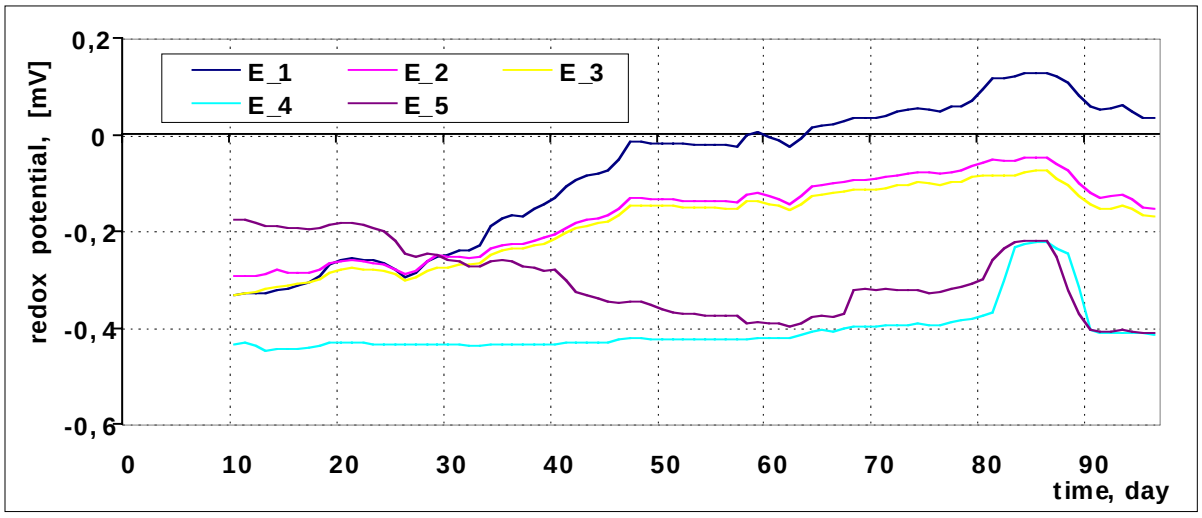


Figure 21 Redox potentials of soil in the bioreactor

7.5.2 Chemical analyses

7.5.2.1 Gas

O_2 and CO_2

Gaseous oxygen concentration data (Figure 22) proved to be successful for maintaining low oxygen content throughout the bioremediation process. High CO_2 concentrations probably resulted from fermentative decomposition of organic matter in sewage sludge.

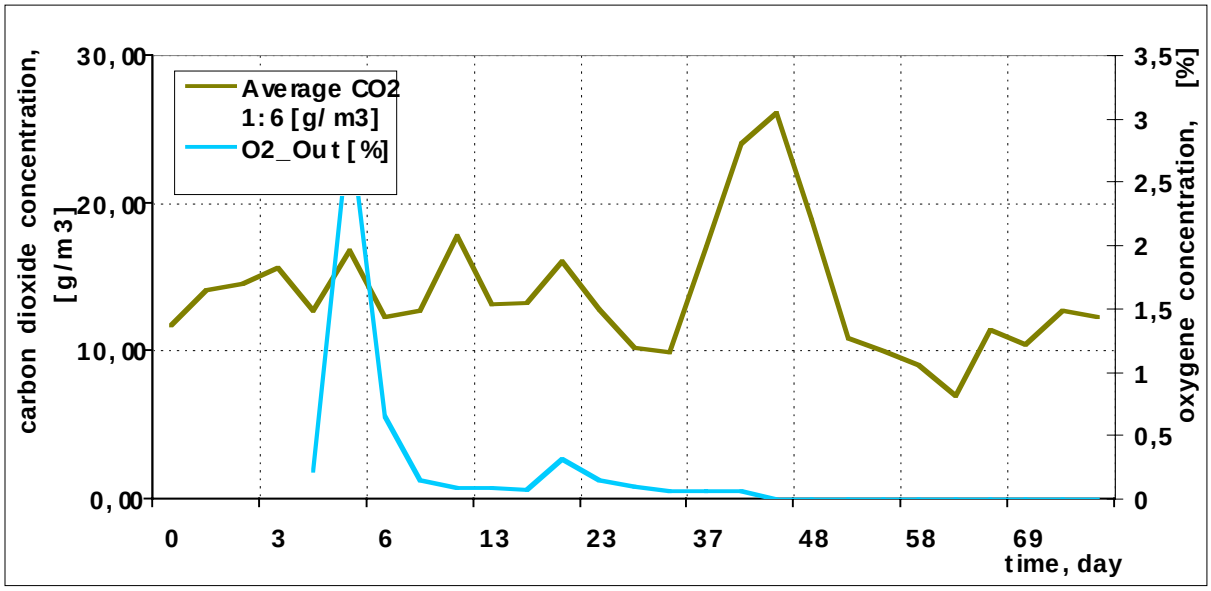


Figure 22. Oxygen and carbon dioxide concentrations in gas in the bioreactor

TCE, DCE, VC and ETH

Before the day 18, only small quantities of daughter products (DCE) were sporadically detected in soil gas samples (Figure 23). Since the 22nd day the DCE concentrations rose quickly with a simultaneous drop in TCE concentrations.

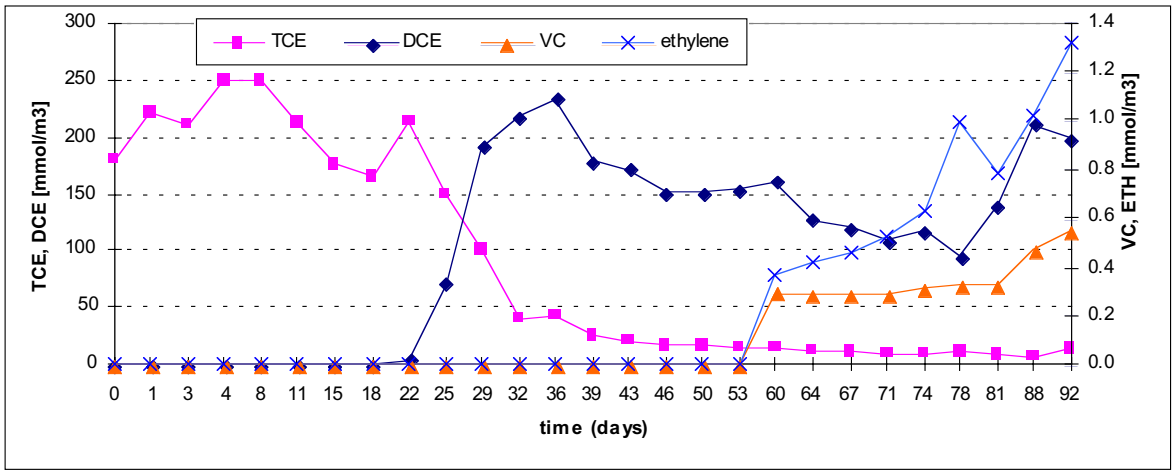


Figure 23. TCE, DCE, VC and ETH concentrations in gaseous phase vs. time during bioremediation test in the bioreactor

Further measurements showed a gradual TCE concentration decrease and subsequent leveling off (after day 50.) and decrease in DCE concentration followed by clear increase after day 78. Presence of VC and ETH was not detected until day 53. Since the day 60 of the process, a new GS-Gastro column, characterized by high resolution of light chlorinated alkanes/alkenes was used for more precise determination of lighter TCE dechlorination products, i.e., VC and ETH. After that, small quantities of daughter products, VC and ETH, were detected in soil gas samples on day 60. (Table 13). The gaseous phase composition is influenced by many processes occurring simultaneously in the bioreactor, including microbial degradation, intra- and inter-phase mass transfer of the TCE and daughter products, etc. Also, all these processes are influenced by temperature inside the bioreactor, giving as the result rather complex relation between concentrations of the bioreactor gas phase components.

Table 13. TCE, DCE, VC and ETH mean concentrations in soil gas as an average of 5 sampling points [mmol/m^3]

Day	TCE	DCE	VC	ETH
0	180.65	0.05	0.00	0.00
1	221.03	0.04	0.00	0.00
3	210.22	0.30	0.00	0.00
4	247.97	0.09	0.00	0.00
8	248.82	0.00	0.00	0.00
11	212.61	0.00	0.00	0.00
15	175.17	0.32	0.00	0.00
18	164.21	0.60	0.00	0.00
22	213.50	3.67	0.00	0.00
25	149.97	71.71	0.00	0.00
29	100.74	190.61	0.00	0.00
32	40.28	217.04	0.00	0.00
36	42.91	233.36	0.00	0.00
39	24.58	178.91	0.00	0.00
43	20.32	171.22	0.00	0.00
46	16.42	151.11	0.00	0.00
50	16.77	151.01	0.00	0.00
53	15.12	154.11	0.00	0.00
60	15.35	160.57	0.29	0.36
64	12.80	127.07	0.29	0.42
67	11.82	117.44	0.28	0.46
71	10.38	109.35	0.29	0.53
74	9.33	116.62	0.31	0.62
78	9.14	94.84	0.33	0.99
81	8.29	139.56	0.33	0.78
88	7.03	210.99	0.47	1.02
92	14.13	198.73	0.55	1.32

Raising the temperature inside the bioreactor caused the increase of TCE and dechlorination product concentrations in the gaseous phase. It cannot be concluded, however, that the temperature increase caused immediate acceleration of the dechlorination process. In the case of TCE and DCE the concentration increase was caused by a phase equilibrium change. Results of further measurements should show whether the observed VC and ETH concentration increase was due to the increased biodegradation rate or physical condition changes.

7.5.2.2 Leachate

Results of the leachate analyses are presented in tables 14 and 15.

Table 14. TCE, DCE, VC and ETH concentrations in leachate [mmol/L]

Day	TCE	DCE	VC	ETH
18	0.6502	0.0116	0	0
25	0.0808	0.9577	0	0
28	0.0103	1.5414	0	0
45	0.0013	1.6449	0	0
60	0.0003	1.2099	0	0
67	0.0005	1.7797	0	0
72	0.0007	2.0696	0	0
86	0.0035	1.5001	0	0

Table 15. Concentrations of selected leachate components [mg/L]

Day	NNH_4 mg/l	NNO_2 mg/l	NNO_3 mg/l	Ntotal mg/l	pH	Cond. mS/cm	PO_4 mg/l	Ptotal mg/l	Cl mg/l	BZT_5 mgO_2/l	ChZT mgO_2/l	TPH mg/l	TPH+pol mg/l
18	48.1	<0.1	<0.2	67.8	7.2	1.91	0.36	0.90	38	28.0	2697	3.85	6.39
25	56.2	<0.1	<0.2	65.2	7.2	1.30	0.17	0.90	126	24.4	2836	5.32	7.59
28					7.3	2.02							
45					6.9								
60	51.9	<0.1	0.40	73.2	6.9	2.20	0.31	3.06	243	21.4	4080	1.13	7.00
67					6.9				252				
72					6.9				248				
86	1.4	<0.1	0.41	65.5	6.8	2.46	0.67	3.10	260	30.1	4575	5.59	8.46

Leachate chemical composition changes depend on both the phase of the process occurring in the bioreactor and on the leachate amount. Moreover, concentrations of TCE and DCE, substances of low boiling temperature largely depend on the temperature inside the bioreactor.

TCE concentration in the leachate depends on TCE concentration in the gaseous phase, whereas no relation was observed in the case of DCE concentration, especially at low temperatures.

At the beginning of the process a slight decrease of pH and then its stabilization at 6.8 was observed. Initial dechlorination progress was reflected by chloride ion concentration increase, and the recorded moderation caused by low temperature was reflected in stabilization of their concentrations.

No significant changes in nutrient concentrations were observed, except a sudden drop of NH_4 ion content.

7.6 Conclusions

The main goal of the project was achieved. A 6 m^3, packed bed, mobile bioreactor to remediate the small amounts of chlorinated solvent contaminated soils was built and tested.

During the test, approximately 4 tons of soil from IETU s backyard, artificially contaminated with 1L (~1640 g) of trichloroethene and amended with sewage sludges from two wastewater treatment plants has been bioremediated under anaerobic

conditions for 90 days. Thermal insulation and supplemental heating of the bioreactor allows for bioremediation progress despite low ambient temperatures.

Results obtained to date, i.e., decreasing TCE concentration, considerable amounts of DCEs, and occurrence of VC and ETH in gas phase, indicate that the stepwise dechlorination of TCE occurs in the bioreactor. Also, increasing amounts of chloride in leachate support this conclusion. Due to complex bioremediation conditions quantitative analyses are difficult to perform. During FY02 a sampling procedure and process model will be created.

From a mechanical point of view, bioreactor operation was smooth and no major obstacles emerged. Nevertheless, some changes in the bioreactor system are planned in order to improve its operation:

- The one-stage reducing valve currently used to depressurize cylinder gases delivered to the reactor should be replaced by a two-stage system to enable safer and more precise operation.

- An adsorber/absorber should be added to the gas circulation system to remove harmful components from off-gases. The amount of off-gases will increase when the anaerobic/aerobic process is tested.

Advantec ADAM modules and the PC based acquisition system were useful. Developed at the IETU, BioReDaq software is capable of data averaging and logging, sensor power control and sensor output signal recalculating. In order to improve the data acquisition the B&K Photoacoustic Gas Monitor Type 1302 should be provided with optical filters enabling "on line" determinations of TCE, DCEs, VC and ethylene concentrations in gases circulating through the bioreactor and to incorporate these data into a computer controlled data acquisition system.

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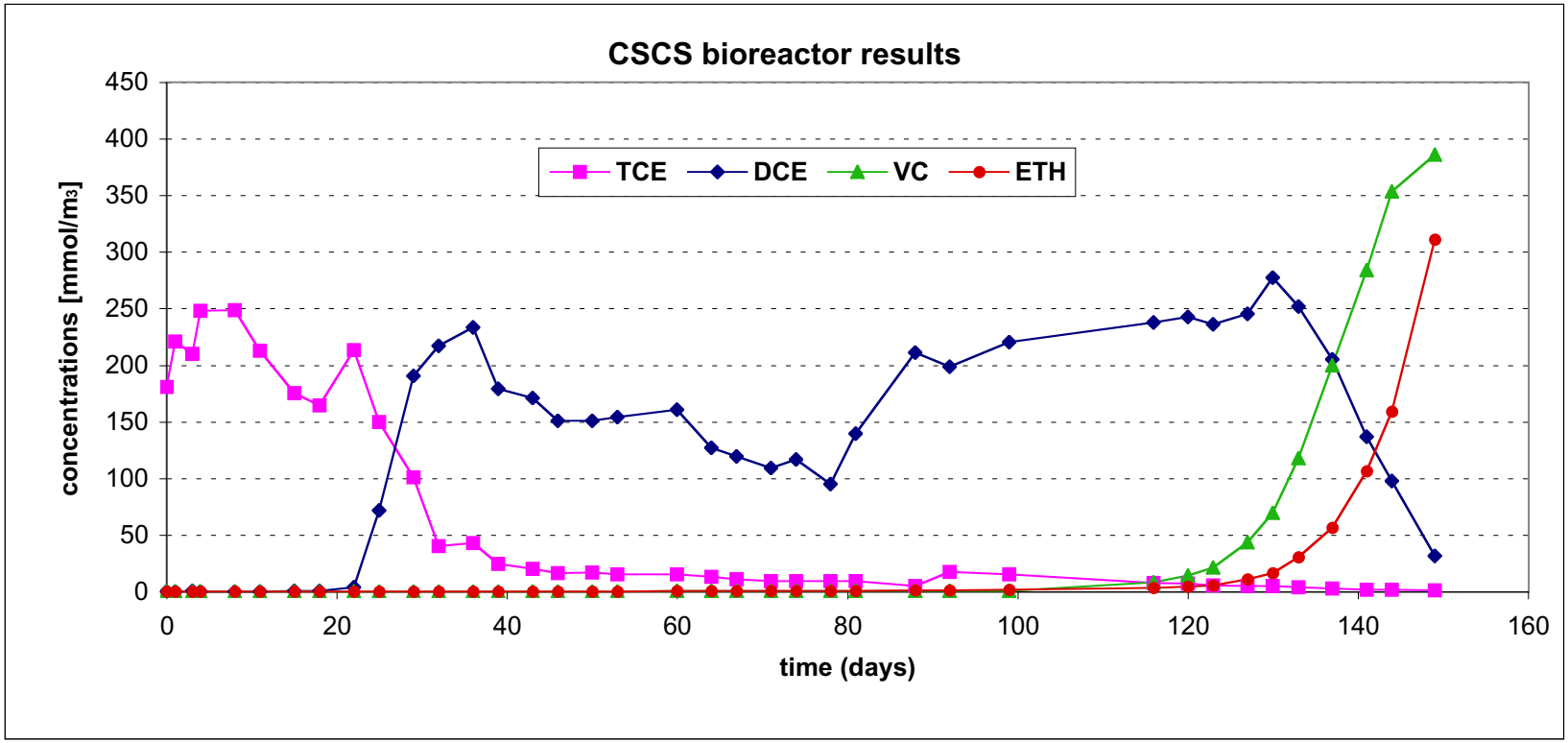
CSCS bioreactor results
TCE
DCE
VC
ETH
concentrations [mmol/m3]
time (days)
0
50
100
150
200
250
300
350
400
450
0
20
40
60
80
100
120
140
160

MONITORING OF ASSISTED NATURAL ATTENUATION

AT THE REFINERY BIOPILE

Summary

The project compared long- (1997-2001) and short-term (2001) data concerning the bioremediation process at the refinery's engineered biopile. Activities in 2001 were carried out with natural aeration and application of mineral NPK fertilizers and the surfactant, Rokafenol N8, to stimulate the removal of recalcitrant hydrocarbons from the biopile soil. Bioremediation with application of these methods and employing indigenous microorganisms is often referred to as monitored natural attenuation (MNA).

The hydrocarbon mass was decreased up to 77.7 %. The bioremediation period was divided into four major stages. The first stage (through the 7th month) was characterized by the considerable decrease of aliphatic hydrocarbons and high microbial activity in the biopile soil. Much higher removals of contaminants were observed in the active section and deep layer than in the passive section and shallow layer of the biopile. These phenomena were explained with intensive aeration and high initial concentrations of contaminants in the active section and deep layer of the biopile. The second bioremediation stage (between the 7th and 21st month) was characterized by the increase of contaminants, which could better be observed in the passive section and shallow layer than in the active section and deep layer of the biopile. The contaminant increase was probably caused by gradual desorption of hydrocarbons from soil particles. The second considerable decrease of contaminants characterized the third stage of bioremediation (between the 21st and 24th month). This decrease was explained with the first treatment of the biopile with the surfactant, Rokafenol N8. Surfactants markedly increase the availability of hydrocarbons to soil microorganisms.

The fourth bioremediation stage began in the 24th month and has lasted till present and included activities conducted in 2001. A considerable decrease of contaminants together with extremely high microbial activity was observed between February and July (39th and 44th month of bioremediation). However, the concentrations of contaminants increased and the microbial activity decreased in October. These seasonal changes were especially well observed in the former active section and deep layer of the biopile. It is believed that intensive mineral NPK fertilization, along with the addition of Rokafenol N8 to the soil, caused the increase of hydrocarbon availability to microorganisms, high microbial activity and high removal of hydrocarbon contaminants from the soil. However, the environmental conditions diminished in autumn months (temperature decreased, high soil moisture maintained) and resulted in the considerable decrease of microbial activity and the increase of hydrocarbon concentrations in October. Possible leakage and movement of contaminants from the non-engineered biopile (during its construction) to the former active section of the engineered biopile also could add to this increase.

The PAH changes also were multistage but less considerable than the changes of aliphatic hydrocarbons during bioremediation. It is noteworthy that the general PAH mass loss was higher than the TPH/TPOC mass loss during the whole bioremediation project.

The conclusion from the Microtox® studies was that the soil from the former active section of the biopile still contained high concentrations of toxic and mutagenic

substances (mainly PAHs), showed acute toxicity and was dangerous from an ecotoxicological point of view. To complete the ecotoxicity data, it is suggested to examine soil from the biopile with the Ames mutagenecity/toxicity test and/or other tests that are more sensitive to mutagenic compounds.

Based on results obtained with the ***Trichophyton ajelloi*** R66 leachate test, the bioremediation process was divided into three stages (<6, 6-9, >9 months). The inhibition of fungal growth was very high through the 6th month of bioremediation and drastically decreased between the 6th and 9th month of the process. During the following months, the inhibition values were low, however, with distinct fluctuations observed. The low inhibition of fungal growth was associated with TPOC concentrations in leachate below 5 mg/L. The results confirm the usefulness of the fungal test in bioremediation studies but mainly for assessment of initial bioremediation stages, during which concentrations of polar compounds in leachate are high. The study indicated the need for finding a fast and easy fungal test employing strains with sensitivity to hydrocarbon contaminants higher than that of ***Trichophyton ajelloi***. It appears that strains of other dermatophyte species may meet this goal.

Keratinolytic and keratinophilic fungi occurred in soil at the engineered biopile with relatively high frequencies. The fungal ability to degrade aliphatic hydrocarbons in the presence of proteins (co-metabolism) can explain this phenomenon. The long-term data obtained with the traditional Vanbreuseghem's method (with incubation at room temperature) showed relationships between the concentration of contaminants (polar compounds, in particular) and fungal frequency. Thus, the data confirmed the utility of the method in bioremediation studies. However, the need appeared to identify fungal species with higher sensitivity to hydrocarbon contaminants in soil and/or another more sensitive method for ecotoxicity/biodiversity assessment of bioremediation. The 4-temperature method provides more information than the traditional method on the fungal community in the biopile soil. It appears that this method, with the hydrocarbon-sensitive species, ***Chrysosporium keratinophilum,*** may meet the goal.

1. Introduction

One of the main activities conducted by the Institute for Ecology of Industrial Areas (IETU), in cooperation with the Department of Energy (DOE), included the construction, operation and evaluation of a biopile at the Czechowice Oil Refinery (COR) in Poland. The purpose of the biopile was to evaluate novel technologies and research approaches to the bioremediation of soil heavily contaminated with petroleum waste. In particular, the DOE/IETU biopile project (1997-1999) compared the bioremediation process under active vs. passive aeration, as well as removal rates of both readily-available-to-microorganisms and residual (remaining, recalcitrant) petroleum hydrocarbons. The addition of mineral fertilizers and the surfactant, Rokafenol N8, to the biopile soil was found to accelerate the bioremediation process (IETU, 1999). The biopile microbial community also was examined. This community was found to consist of bacteria and microscopic fungi highly specialized in hydrocarbon biodegradation. Special attention was paid to examination of acidophilic microorganisms and keratinolytic/keratinophilic

fungi in the biopile (Ulfig ***et al.***, 2000). The main biopile project was completed in 1999. The site currently exists as a green space within the COR site. However, the biopile soil and leachate still contain residual and toxic hydrocarbon contaminants and, therefore, require further treatment and monitoring. The present project concerns these activities.

Three hydrocarbon waste lagoons presently exist within the COR site. The DOE/IETU engineered biopile was designed and constructed in 1997 in the smallest lagoon. The bioremediation process has been monitored continuously since construction. Successful bioremediation results obtained by the DOE/IETU staff inspired the COR management, in close cooperation with IETU specialists, to design and build another biopile to remediate soil mixed with petroleum hydrocarbon waste in the second refinery lagoon. The design for reclamation of the second lagoon was prepared by Worsztynowicz ***et al.*** (2001). Construction of this new biopile was complete in November 2001. The new biopile uses a simple and cost-effective ***in situ*** bioremediation technology. It has a drainage system and the dolomite/organic soil layer covers the soil heavily contaminated with hydrocarbon waste, but the bioremediation process is presumably carried out under very passive (almost anaerobic) conditions. In contrast to the DOE/IETU engineered biopile, the new biopile will be called, hereafter, the non-engineered biopile. The project presents preliminary results related to this biopile.

2. Project Goal & Activities

The primary goal of the present project was to compare long- and short-term data from monitoring the bioremediation process at the engineered biopile. Ecotoxicity and biodiversity in soil and leachate from the biopile after long-term bioremediation also was determined. An additional goal of the project was a preliminary comparison of bioremediation processes at the engineered and non-engineered refinery biopiles.

Bioremediation activities in 1997-1999 were described in detail in a previous report (IETU, 1999). Activities in 2001 were conducted with natural aeration and application of mineral NPK fertilizers and Rokafenol N8 to stimulate the removal process of recalcitrant hydrocarbons from biopile soil. Bioremediation with application of these additives and employing indigenous microorganisms often is referred to as monitored natural attenuation (MNA).

The project consisted of the following major tasks:

- Performance of four sampling campaigns at the engineered biopile and one sampling campaign at the non-engineered biopile;
- NPK fertilization of the engineered biopile;
- Treatment of the former active section of the biopile with the surfactant, Rokafenol N8;

- Analysis of long- (1997-2001) and short-term (2001) data concerning the bioremediation process at the engineered biopile;
- Evaluation of climatic conditions at the COR site in 2001;
- Ecotoxicity assessment of soil and leachate from the engineered biopile;
- Physiological, ecological and ecotoxicological characterization of the keratinolytic and keratinophilic fungal community at the engineered biopile; and
- Preliminary comparison of bioremediation conditions at the engineered biopile and non-engineered biopile.

3. Material & Methods

3.1. Sampling & analyses

The sampling strategy was similar to that applied in a previous project (IETU, 1999). In 2001, four sampling campaigns (in February, May, July, and October) were performed at the engineered biopile while one sampling campaign (in November) was conducted at the non-engineered biopile. The total number of sampling locations at each biopile was twenty-three. Sampling location distributions at the engineered biopile and the non-engineered biopile are presented in figures 1 and 2, respectively. Soil was sampled (ca. 1 kg) from shallow (ca. 30-40 cm of depth) and deep (ca. 80-100 cm of depth) layers of the biopiles. Leachate samples (ca. 3 L) were collected from leachate collecting wells. Each soil and leachate sample was examined for the following physico-chemical parameters: TPH – total petroleum hydrocarbons (non-polar aliphatic hydrocarbons); TPOC – total petroleum organic carbon (polar and non-polar aliphatic petroleum compounds); pH in H_2O; pH in 1 M KCl; conductivity; $N\text{-}NH_4$; $N\text{-}NO_2$; $N\text{-}NO_3$ and PO_4. The parameter, POLAR (concentration of polar aliphatic compounds = TPOC–TPH), also was used. The parameter PAH (= PAHs Total) was calculated based on the sum of the concentrations of the following polycyclic aromatic hydrocarbons: naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,i)perylene, dibenzo(a,h)anthracene, and indeno(1,2,3-cd)pyrene. Concentrations of PAHs were determined at the beginning and end of the project (during the first and fourth sampling campaigns) at the engineered biopile and during one sampling campaign (in November 2001) at the non-engineered biopile. Soil moisture also was measured. All results were presented with regard to soil dry weight (d.w.).

During the first sampling campaign additional soil analyses were performed for determination of NPK fertilization doses at the engineered biopile. The biopile surface was divided into four subsections (Figure 1). At each sampling location, within each subsection additional soil samples from shallow and deep layers were collected, dried, thoroughly mixed together and analyzed for the following parameters: total phosphorus (P_{TOT}), available phosphorus (P_2O_5), available potassium (K_2O), total organic carbon

(TOC), and total nitrogen (N_{TOT}). Methods, standards and references used in physico-chemical analyses are presented in Table 1.

Microbiological analyses performed on each sample collected were as follows: total number of bacterial cells with the epifluorescence DAPI method; total number of fungal propagules with the epifluorescence Calcafluor white (CW) method; dehydrogenase activity with the TTC method; and the number of naphthalene- and crude petroleum-degrading microorganisms with the MPN (Most Probable Number) technique combined with the enrichment method in Biolog® microplates at 23 and 37 ^{o}C.

The DAPI epifluorescence method was that of Kepner & Pratt (1994). The CW epifluorescence method was adapted from medical mycology (Sparkes ***et al.,*** 1994). An "Axioscop" microscope by Carl Zeiss Opton (Germany) was used for direct enumeration of microorganisms in soil and leachate with epifluorescence methods. The TTC method for measurement of dehydrogenase activity in soil was that of Alef (1996). The MPN technique in Biolog® microplates was used for enumeration of naphthalene- and petroleum-degrading microorganisms in soil and leachate at 23 and 37 ^{o}C. Soil and leachate dilutions were made in tubes with 1 % NaCl solution. Each MPN well in a Biolog® microplate contained 100 µl of liquid mineral medium (MM) and 10 µl of autoclaved crude petroleum or a small crystal of autoclaved naphthalene. MPN wells inoculated with appropriate sample dilutions were incubated in the dark for 2 weeks at 23 or 37 ^{o}C. After incubation, growth of microorganisms in each MPN well was confirmed by inoculation on SMA (Standard Methods Agar) medium. MPNs were calculated with the equation given by EPA-670/9-75-006 (1975).

3.2. NPK fertilization of the engineered biopile

Based on results presented in Table 2, mineral NPK fertilization doses were determined for four subsections of the engineered biopile. Mixed soil samples I and II were taken from two subsections of the former active biopile section while mixed samples III and IV were collected from two subsections of the former passive biopile section (Figure 1). The four subsections (I-IV) markedly differed in values of physico-chemical parameters measured. In general, soils showed average and high concentrations of available phosphorus and potassium, but nitrogen concentrations were low. Due to the high carbon concentrations and low nitrogen contents C:N ratios were high. Therefore, requirements of plants and soil microorganisms for nitrogen were extremely high. A computer program routinely used in agriculture calculated NPK fertilization doses for each biopile subsection as well as for the entire biopile. Total biopile NPK requirements were determined as follows: 37.3 kg of NH_4NO_3 (34 % N), 5 kg of triple super-phosphate (46 % P_2O_5) and 3.7 kg of KCl (60 % K_2O) per hectare. Each biopile subsection was fertilized individually in accordance with its NPK requirements. These NPK doses were divided into three portions (40, 30 and 30 % of the total NPK doses). The first portion of mineral fertilizers was spread over the biopile surface in April. The second and third fertilizations were performed in June and August, respectively. This three-part mineral fertilization campaign was designed to avoid destruction of plants and microflora on the biopile surface due to excess mineral salts and to ensure the gradual salt dissolution and distribution throughout the biopile profile.

3.3. Rokafenol N8 treatment of the engineered biopile

In a previous study (IETU, 1999), the synthetic surfactant, Rokafenol N8, considerably increased the removal rate of hydrocarbon contaminants from the engineered biopile. Therefore, it was decided to use this surfactant again in 2001. The former active section of the biopile (Figure 1) was selected for this treatment, because of its relatively high hydrocarbon concentrations. The dose established was 134 L per hectare. This dose was divided into four equal portions and spread over the treated surface four times within a month at one-weak intervals to avoid destruction of plants and microflora on the biopile surface. The surfactant was applied to the biopile in water solution (2 % w/w).

3.4. Ecotoxicity assessment

The Microtox® Test System was used for determination of soil and leachate ecotoxicity after a long-term bioremediation at the biopile. This testing was performed at the Department of Environmental Engineering, Polytechnic Institute in Kraków (head: Dr. Beata Cwalina).

The Microtox® Test System is based upon the use of luminescent bacteria, specifically the strain ***Vibrio fischeri*** NRRL B-11177, to measure toxicity in environmental samples. When properly grown, luminescent bacteria produce light as a by-product of cellular respiration. Bacterial bioluminescence is tied directly to cell respiration, and any inhibition of cellular activity (toxicity) results in a decreased rate of respiration and a corresponding decrease in luminescence rate. Bacterial bioluminescence has proven to be a convenient measure of cellular metabolism and consequently, a reliable biosensor for measuring toxic conditions. Strain 11177 originally was chosen for the acute and chronic tests because it displayed a high sensitivity to a broad range of chemicals. The Microtox® Test has achieved official “Standards Status” in several countries, including an ASTM Standard (D-5660) in the US. The final ISO Draft (11348-3) entitled “Water quality-determination of the inhibitory effect of water samples on the light emission of ***Vibrio fischeri*** (Luminescent Bacteria) test” has been approved.

The Microtox® Test System was used for ecotoxicity assessment of soil and leachate samples from the engineered biopile during the third sampling campaign (July 2001). The biopile area was divided into eight subsections (1-8). Subsections included the following locations and layers: subsection 1 (locations 1, 2, 7, 8, 9, 10 – deep layer); subsection 2 (locations 1, 2, 7, 8, 9, 10 – shallow layer); subsection 3 (locations 3, 4, 5, 6, 11, 12 – deep layer); subsection 4 (locations 3, 4, 5, 6, 11, 12 – shallow layer); subsection 5 (locations 13, 14, 18, 19, 20 21 – deep layer); subsection 6 (locations 13, 14, 18, 19, 20 21 – shallow layer); subsection 7 (locations 15, 16, 17, 22, 23 – deep layer); and subsection 8 (locations 15, 16, 17, 22, 23 – shallow layer). Subsections were established with regard to the physico-chemical and microbiological conditions at the biopile. Within each subsection additional soil samples were collected, dried and mixed together thoroughly. Mixed samples, together with leachate from the leachate collecting well were examined for TPH, TPOC, PAH, pH in H_2O, pH in 1 M KCl, conductivity, moisture, N-NO_2, N-NO_3, N-NH_4, magnesium, calcium, PO_4, total phosphorus (P_{TOT}), available phosphorus (P_2O_5), available potassium (K_2O), total organic carbon (TOC),

total nitrogen (N_{TOT}), S-SO_4, total sulfur (S_{TOT}), and heavy metals (Cd, Pb, Zn, Cu, Cr, Ni, Co, Mn, Fe, Hg, and As). Heavy metals were measured with AAS and ICP techniques (EPA 6010B). Mixed samples were delivered to the Department of Environmental Engineering, Polytechnic Institute in Krakow for ecotoxicity examination.

For ecotoxicity assessment, 22 % NaCl solution was added to the leachate sample to obtain the final concentration of 2% NaCl. Twenty grams from each mixed soil sample were mixed with 100 mL of deionized water or with 100 mL of 5 % ethanol solution and the mixtures were shaken for 24 hrs. Mixtures then were left for 6 hrs for sedimentation of soil particles. 22 % NaCl solution was added to the supernatant to obtain the final concentration of 2% NaCl (Symons & Sims, 1988; Skacel ***et al.***, 1993; Demuth ***et al.***, 1993).

Toxicity measurements were performed after 5, 15 and 30 minutes of incubation at 15 ^{o}C, using increasing dilutions of soil water extracts. The EC_{50} values obtained with the Microtox® Test System were interpreted as follows: The EC_{50} = 20 % means that a sample containing 20 % of an extract and 80 % of a solvent causes mortality in 50 % of bacteria. In this interpretation, the lower EC_{50} percentage the higher toxicity of the examined sample. If the EC_{50} value is >100 %, it means that the sample shows no toxicity.

The fungal ecotoxicity test for leachate was described by Ulfig ***et al.*** (1998). The test uses the ***Trichophyton ajelloi*** R66 strain. Results obtained with this strain are presented in this report.

3.5. Characterization of the fungal community

In the present project, physiological, ecological (biodiversity), toxicological and taxonomic characterization of the fungal community at the engineered biopile was continued. These studies addressed the composition of keratinolytic and keratinophilic fungi resistant to actidione (cycloheximide) during bioremediation at the biopile. Keratinolytic fungi have an ability to vigorously digest keratinous substrata whereas keratinophilic fungi display a weaker affinity for these substrata, decomposing mainly the non-protein components or the products of keratin degradation. The resistance of these fungi to actidione allows easy isolation from environmental samples.

The studies were composed of three parts. The first and major part concerned the long- and short-term changes in the qualitative and quantitative composition of keratinolytic and keratinophilic fungi during bioremediation at the engineered biopile. In these studies, the traditional Vanbreuseghem's method (1952), with incubation of soil samples in the dark at room temperature (ca. 23 ^{o}C) for four months, was employed. The indices of fungal growth were as follows: number of strains (NST), number of species (NSP), and the frequencies of isolated species. The most common species was the geophilic dermatophyte ***Trichophyton ajelloi***. Its frequency was named TAJ.

The ecotoxicological and biodiversity aspects of mycological data were explained with two methods. In the first method, relationships between the fungal indices and the

concentrations of TPH, TPOC, POLAR, and PAHs were found and statistically evaluated with the ANOVA (Analysis of Variance) method. The second method was based upon the following rule: At first, the mycological variables, i.e., fungal indices and frequencies, were grouped in one file together with a selected environmental parameter. The grouped data were sorted using the sort data option in the Excel program with the ascending sorting order and the environmental parameter as a key variable. The sorted variables were then recalculated to obtain the cumulative frequencies of fungal indices and cumulative frequency of the selected environmental parameter. To compare different parameters, this procedure was repeated for each physico-chemical key variable examined. The results were plotted putting the cumulative frequencies of physico-chemical parameters on the X-axis and the cumulative frequencies of fungal indices on the Y-axis. Another option of the method was to plot the absolute values of the selected environmental parameter on the X-axis and the cumulative frequencies of fungal indices on the Y-axis. Figure 3 displays this option as a hypothetical example on the influence of an environmental factor on three fungal species. In this example, the environmental factor does not influence the frequency of species 1 (neutral relation). The inhibition and stimulative effects of the factor are evident in the cases of species 3 and 2, respectively.

The second part of the fungal studies began in February 2001 and addressed the qualitative and quantitative composition of keratinolytic and keratinophilic fungi growing at four temperatures. In this part, the modification of the Vanbreuseghem's method relied on the incubation of soil samples at 23, 29, 33 and 37 ^{o}C (Ulfig ***et al.***, 2001). The temperatures 29, 33 and 37 ^{o}C allowed isolation of many fungal strains that grow slowly at room temperature (23 ^{o}C). Fungal growth at these temperatures is shown in Figure 4. In this part of fungal studies, some additional terms were used: TOTAL (=NST_{TOT}; the total number of fungal strains), $TOTAL_{23}$ (=NST_{23}; the number of strains isolated at 23 ^{o}C), $TOTAL_{29}$ (=NST_{29}; the number of strains isolated at 29 ^{o}C), $TOTAL_{33}$ (=NST_{33}; the number of strains isolated at 33 ^{o}C), and $TOTAL_{37}$ (=NST_{37}; the number of strains isolated at 37 ^{o}C). The terms for the numbers of isolates of fungal species also were used. The most important were those of species predominating in the biopile soil, i.e., ***Trichophyton ajelloi*** (TAJ) and ***Chrysosporium keratinophilum*** (CKER). In the analysis of 4-temperature data, PCA (Principal Component Analysis) and ANOVA methods were employed.

The third part of fungal studies started in 2000 and addressed the physiological and morphological properties of ***Microsporum ripariae***-like strains isolated from the engineered biopile. ***Microsporum ripariae*** is a rare fungus (only one strain has been recorded) similar to ***Microsporum fulvum***, with an ability to degrade hydrocarbons in the presence of proteins (unpublished data). Micro- and macro-morphological studies of ***Microsporum ripariae***-like strains have been performed this year. These studies will be continued next year. The ***Microsporum ripariae***-like strains will be examined from a biomolecular point of view (specifically, sequences of the ITS1, 5.8 S and ITS r-DNA regions), in cooperation with the University of Rovira and Virgili at Reus (Spain) and the Centraalbureau voor Schimmelcultures at Baarn (Netherlands). Final results from the third part of the fungal studies will be presented next year.

3.6. Climatic studies

The main goal of climatic studies was to analyze the thermal and moisture conditions in soil at the engineered biopile for the period of January-September 2001. The studies were based on routine observations collected at the Bielsko-Biala meteorological station located close to the refinery area. The parameters used in the studies were the following:

1. Daily mean temperature (T), air relative moisture (F) and daily sums of precipitation (P);

2. Noon (12^{00} GMT) and daily mean soil temperature at 4 depths: 5, 10, 20, and 50 cm;

3. State of soil surface (SG) under snow and snow-absence conditions (according to the international scale) at 6^{00}, 12^{00}, and 18^{00} GMT; and

4. Soil moisture index (WW) computed with the following formula (Sztyler; in: Ulfig ***et al.***, 1996):

$$WW = (1/1+\Sigma i)*[(P_1 - E_1)*\Sigma i\ (P_i - E_i)]$$

where:

i = 1, 2,....., n consecutive days; $1/1+\Sigma_i = 0.0179$ (for n = 10 consecutive days),

$E = f(t)*(25 + T)^2*(1- F/100)$ - dryness power of the air according to the Ivanov's formula (Okolowicz, 1969),

f (t) = 0.0059 (for daily mean values), and

P = total daily precipitation (mm).

Soil moisture index (WW) approximately expresses the magnitude of excess or deficit of water reaching the earth surface (P) in relation to the dryness air power in the end of a given (consecutive) time period (e.g., 10 days). The computation of WW-index values was performed for the period of April-September, because of more or less constant snow cover during the period of January-March 2001. This analysis of meteorological data resulted in the development of monthly T, F, P, WW and SG values, as well as soil temperature (T_G) at the depth of 5-50 cm. The analysis also included the WW and SG variations at noon (12^{00} GMT) and daily mean soil temperature as a function of depth and the state of soil moisture.

3.7. Statistical analyses

Statistical analyses of results obtained were performed with the STATISTICA 5.1 for Windows program. Results were processed with parametric methods. The analysis of variance (ANOVA) was a major method in evaluation of statistical differences between variables.

4. Results

4.1. General assessment of physico-chemical and microbiological data

Descriptive statistics for selected (most important) physico-chemical and microbiological parameters for the whole bioremediation period (1997-2001) at the engineered biopile are presented in tables 3 and 4, respectively. Both sorts of data displayed high variability.

Simple linear correlations between selected physico-chemical parameters (including dehydrogenase activity) are presented in Table 5. The highest correlations were obtained between the concentrations of petroleum-originating contaminants such as TPOC, TPH, and POLAR (r coefficients ≤ 0.97). This is understandable, since these parameters were measured from the same CCl_4 extracts (TPH and TPOC) or calculated from each other (POLAR). However, the correlations between the above-mentioned parameters and PAHs were much lower ($r \leq 0.56$). The correlations between pH in H_2O, moisture and dehydrogenase activity (TTC) with the concentrations of hydrocarbon contaminants were very low but statistically significant. No statistically significant correlations between TTC and the concentrations of hydrocarbons were found. The only statistically significant, but low, correlation ($r = 0.27$) was between TTC and moisture.

The correlations between microbiological and selected physico-chemical parameters (including dehydrogenase activity) are presented in Table 6. Negative correlations were found between the number of bacterial cells (DAPI) with TPH, TPOC, POLAR, PAH and TTC and positive correlations between the total number of fungal propagules (CW) with TPH, TPOC and POLAR. The positive correlation between the most probable number (MPN) of naphthalene-degrading microorganisms at 23 ^{o}C ($NAPH_{23}$) and the total concentration of PAHs ($r=0.21$) also was demonstrated.

Figures 5 and 6 illustrate the means for selected physico-chemical parameters for passive/active and shallow/deep sections/layers of the engineered biopile, respectively. The mean concentrations of hydrocarbons (TPH, TPOC, POLAR, and PAHs) and the mean dehydrogenase activity (TTC) were distinctly higher for the active section and deep layer than the passive section and shallow layer of the biopile. The differences were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$).

4.2. Changes in selected parameters during bioremediation

The pH, moisture and dehydrogenase activity (TTC) means for sampling campaigns in 1997-2001 are presented in Table 7. These parameters fluctuated, to a high degree, during the bioremediation period. The mean soil pH ranged between 6.41-7.28, while the ranges for the moisture and dehydrogenase activity means were 9.36-21 % and 5.34-96.21 mg TPF/g d.w., respectively. In February and May 2001, the TTC means were rather low. However, the dehydrogenase activity considerably increased in July. The mean, 96.21 mg TPF/g d.w., was the highest during the bioremediation period. However, the microbial activity considerably decreased in October 2001. The changes

were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$).

The mean concentrations of TPH, TPOC, POLAR, and PAHs for sampling campaigns in 1997-2001 are presented in Table 8. Figure 7 illustrates the TPH/TPOC changes with standard errors and standard deviations shown. Generally, during the first stage of bioremediation (through the 7th month of the process) a considerable decrease of TPH/TPOC was observed. Subsequently, a small increase of these contaminants was noticed between the 7th and 21st months of bioremediation. This time period was the second stage of bioremediation at the biopile. The second considerable decrease of hydrocarbon contaminants was between the 21st and 24th months of bioremediation. This relatively short time period was the third stage of bioremediation. The fourth stage began in the 24th month and lasted until present. This stage was characterized by the relatively low concentrations of hydrocarbon contaminants with, however, distinct fluctuations observed. In 2001, the concentrations of polar compounds (POLAR) were much lower than previous concentrations. A distinct decrease of the TPH/TPOC means was observed between the first (February) and third (July) sampling campaigns (between the 39th and 44th months of bioremediation). However, the hydrocarbon concentrations markedly increased in October 2001 (47th month of bioremediation). The means calculated for October even slightly exceeded the means for the first sampling campaign in February. However, these differences were not statistically significant.

Changes in the mean TPH/TPOC concentrations in passive/active sections and shallow/deep layers during bioremediation at the engineered biopile are illustrated in figures 8-11, respectively. In comparison with the general TPH/TPOC changes (Figure 7), the first-stage TPH/TPOC decrease lasted longer (till the 9th month of bioremediation) in the passive section of the biopile (Figure 8). Besides, a distinct increase of hydrocarbon concentrations was observed during the second stage of bioremediation (between the 9th and 18th months of the process). No particular changes in the concentrations of hydrocarbon contaminants were recorded from the 24th month up to present. In comparison with the general TPH/TPOC changes and with the changes in the passive section, distinctly higher fluctuations of petroleum contaminants were noticed in the active section of the biopile (Figure 9). The fluctuations especially addressed the fourth stage of bioremediation. In February 2001, the TPH/TPOC means were markedly higher than at the 24th month of the process. However, the concentrations of hydrocarbon contaminants considerably decreased between the first (February) and third (July) sampling campaigns to increase in October to the values comparable to those from February.

In the shallow layer of the engineered biopile (Figure 10), the first-stage TPH/TPOC decrease lasted till the 9th month of bioremediation. However, the second-stage increase of hydrocarbon contaminants was high. Therefore, the decrease of these contaminants from the 21st to the 24th month of bioremediation was particularly well observed. In 2001, no particular changes in the concentrations of hydrocarbons took place between February-July. In October, an increase of these contaminants was clearly observed. In the deep layer of the biopile (Figure 11), the first-stage decrease

lasted till the 7th month of bioremediation. The second-stage increase and the third-stage decrease of hydrocarbon contaminants were not so clear as in the shallow layer of the biopile. In 2001, a considerable decrease of hydrocarbon contaminants was observed during February-July. In October, however, the concentration of contaminants considerably increased.

4.3. Hydrocarbon mass losses and removal rates

Hydrocarbon mass losses (%) and removal rates (g or mg/kg d.w./month) for the entire bioremediation project (1997-2001) at the engineered biopile are presented in Table 9.

The TPH and TPOC hydrocarbon mass losses for the whole biopile in 1997-2001 were 55.65 and 61.41%, respectively. The PAH mass loss was the highest (65.43%). The hydrocarbon mass losses were much higher in the passive section and deep layer than in the active section and shallow layer of the biopile. The differences were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$).

The TPH and TPOC monthly removal rates were 0.4 and 0.62 g/kg d.w./month, respectively. The removal rates for the active section and deep layer were higher than the removal rates for the passive section and shallow layer of the biopile. The differences were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$).

4.4. Mycological data

Data on the incidence of keratinolytic and keratinophilic fungi at the engineered biopile in 1997-2001 are summarized in Table 10. The traditional Vanbreuseghem's method, with incubation of soil samples at ca. 23 ^{o}C (room temperature) was employed to obtain the data. Altogether, 346 fungal strains, belonging to 13 species, were isolated and identified. Among the species identified, the soil (geophilic) dermatophyte ***Trichophyton ajelloi***, sometimes producing its sexual state (ascomata, teleomorph) ***Arthroderma uncinatum***, predominated with a frequency 78.3 %. The other species were sporadically detected in the soil. The frequencies of these species were less than 5.5 %. Figure 12 displays multi-celled and smooth-walled macroconidia of ***Trichophyton ajelloi***. In contrast to ***Trichophyton*** species, ***Microsporum*** species, for instance ***Microsporum gypseum*** (Figure 13), produce multi-celled macroconidia with echinulate or warty walls. This characteristic allows easy distinction between the species from both genera.

The means for mycological parameters (TAJ, NST, and NSP) were higher in the passive section than in the active section of the biopile (Table 11). These differences were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$). No statistically significant differences were found between the means for mycological parameters between the shallow and deep layers of the engineered biopile.

Figure 14 shows the means, standard errors and deviations for the concentrations of hydrocarbons (Y-axis) in soil samples with or without the growth of the predominating dermatophyte species ***Trichophyton ajelloi*** (X-axis). The means for all hydrocarbon parameters were much lower in samples in which the growth of the above-mentioned

species was observed. The differences were found to be statistically significant when analyzed with the ANOVA method ($p \leq 0.05$). The highest F coefficient was noticed for the TPOC difference.

Figure 15 illustrates another type of the ANOVA analysis of the obtained data. The mean frequencies of ***Trichophyton ajelloi*** gradually decreased with increasing TPOC with, however, high standard errors and deviations observed. The highest TAJ frequencies were observed at TPOCs lower than 15-g/kg d.w., while no fungal strains were isolated from soil samples with TPOCs higher than 100 g/kg d.w. (>10 % w/w d.w.). The general ANOVA effect for the data was statistically significant ($p \leq 0.05$). Another significant factor that influenced the growth of this species and other keratinolytic/keratinophilic fungi in soil was pH (Figure 16). In general, the number of isolates and species increased with increasing pH. No fungi were observed at pH below 3. At pH between 3-5, the numbers of isolates and species were much lower than the numbers of isolates and species at pH over 5. ***Trichophyton ajelloi*** did not occur in soil samples with pH below 4. However, the differences in the qualitative and quantitative composition of keratinolytic and keratinophilic fungi at pH over 5 (values most frequently observed during bioremediation) were not statistically significant.

It results from the plots in Figure 17 that TPOCs, together with PAHs and TTCs, showed the inhibition effects toward the growth of ***Trichophyton ajelloi*** in soil samples from the engineered biopile. The inhibition effect of non-polar aliphatic hydrocarbons (TPHs) on the fungal community, specifically on ***Trichophyton ajelloi***, was not so evident. Based on the plots, moisture and pH did not affect the occurrence of the fungus at the biopile.

The means of fungal indices (TAJ, NST, and NSP) for sampling campaigns at the biopile in 1997-2001 are presented in Table 12. Generally, the mean TAJ and NST values fluctuated, to a high degree, during the bioremediation period. The highest mean NST values were observed at the 7^{th}, 24^{th} and 39^{th} months of bioremediation. The number of species (NSP) ranged between 3-7 during the whole bioremediation period. The general ANOVA effects were statistically significant ($p \leq 0.05$) for all fungal parameters examined.

During the first sampling campaign in 2001, the growth of keratinolytic and keratinophilic fungi in soil from the engineered biopile at four temperatures (23, 29, 33, and 37 ^{o}C) was examined. The results of this study are summarized in Table 13. Altogether, 191 fungal strains, belonging to 11 species, were isolated and identified. ***Trichophyton ajelloi*** (60 strains) with its teleomorph ***Arthroderma uncinatum*** (3 strains), ***Chrysosporium keratinophilum*** (50 strains), ***Pseudallescheria boydii*** (21 strains), ***Chrysosporium*** anamorph ***Aphanoascus clathratus*** (14 strains), ***Chrysosporium queenslandicum*** (14 strains), ***Chrysosporium*** anamorph of ***Aphanoascus reticulisporus/fulvescens*** (12 strains) with one of its teleomorph ***Aphanoascus reticulisporus*** (4 strains) predominated in soil samples from the biopile. Other species sporadically occurred in the samples. At each temperature, different qualitative and quantitative compositions of the examined fungi were observed. At 23 and 29 ^{o}C, ***Trichophyton ajelloi*** (34 strains) along with ***Chrysosporium keratinophilum*** (9 strains) prevailed. At 33 ^{o}C, the composition of the

fungal community changed drastically. ***Chrysosporium keratinophilum*** (19 strains), ***Pseudallescheria boydii*** (13 strains), ***Chrysosporium*** anamorph of ***Aphanoascus clathratus*** (10 strains) and ***Chrysosporium queenslandicum*** (10 strains) prevailed at this temperature. Among the predominated species, ***Pseudallescheria boydii*** was the only keratinophilic species. The other species showed strong keratinolytic properties ***in vitro***. At 37 ^{o}C, the keratinophilic species ***Pseudallescheria boydii*** (8 strains) predominated in the samples. The highest number of strains (NST) was isolated at 29 ^{o}C (NST_{29} = 62) and the lowest at 37 ^{o}C (NST_{37} = 14). The number of species (NSP) at 23 and 29 ^{o}C was the highest (7 species). The lowest number of species was identified at 33 and 37 ^{o}C (5 species). The number of strains was markedly higher in the former passive section than in the former active section of the biopile while the NST values in the deep and shallow layers of the biopile were quite similar (Table 14). The numbers of species (NSP) in the former passive/active sections and in the shallow/deep layers of the biopile ranged between 8 and 11.

The data were first analyzed with the PCA (Principal Components Analysis) method. The results are shown in Figure 18. The distribution of mycological variables indicated that some of the variables, i.e., NST_{TOT}, NST_{29}, NST_{23}, TAJ, and CKER were especially "sensitive" to the concentrations of TPH, TPOC and PAHs in soil at the biopile. These sensitive variables were selected for further studies. Figure 19 illustrates the relations between the number of strains (NST_{TOT}, TAJ, and CKER) and the TPOC concentrations (ranges) in soil samples. All quantitative fungal variables decreased with increasing TPOC. However, the number of ***Chrysosporium keratinophilum*** strains (CKER) reacted to the concentrations of contaminants with the highest sensitivity. The reaction of ***Chrysosporium keratinophilum*** to TPOCs in soil was more sensitive than the reaction of ***Trichophyton ajelloi*** to these contaminants.

4.5. Ecotoxicity assessment

The physico-chemical characteristics of mixed soil samples and leachate from the engineered biopile (collected during the third sampling campaign) are presented in tables 15-17. It is noteworthy that the highest TPOC and PAH concentrations, including the concentration of the most toxic component, benzo(a)pyrene, were observed in sample 5.

The toxicity data obtained with the Microtox® Test System are summarized in Table 18. No toxicity was noticed in water extracts of the samples examined. In the extracts in 5 % ethanol, the toxicity only was observed in samples 5 and 6. The toxicity was higher in sample 5 than in sample 6. If pH of examined samples ranges between 4.6-9.3, the reaction does not affect the light emitted by bacteria in the absence of toxic substances (Villaescusa ***et al.***, 1997). In samples 5 and 6, in which the toxicity was observed, the pH values were in this admissible range. The analysis of the plots in Figure 20 indicates that the toxicity increased with increasing incubation time in samples 5 and 6.

Figure 21 illustrates relationships between the inhibition of ***Trichophyton ajelloi*** R66 linear growth by TPOCs in leachates and the incubation time in days. It is clear that the inhibition decreases with increasing incubation time. The most adequate incubation time

for evaluation of the inhibition effect of TPOCs in leachate on fungal growth is 14 days. Subsequently, Figure 22 illustrates the relationships between the inhibition of fungal growth and the increasing dilutions of three leachates. It is observed that the inhibition values decrease with increasing dilutions in leachates A and C. In leachate B, however, the inhibition of fungal growth at 50 % dilution (50 % of leachate + 50 % of medium) was slightly higher than the inhibition caused by the leachate without dilution (100 %). There was no simple relationship between the inhibition of the linear growth of the ***Trichophyton ajelloi*** strain R66 by TPOCs in leachate and the bioremediation time in months (Figure 23). The inhibition values showed high variability during bioremediation at the engineered biopile. Based on the results obtained, however, the bioremediation process can be divided into three stages (<6, 6-9, >9 months). The inhibition of fungal growth was high to the sixth month of bioremediation. Subsequently, the inhibition values decreased considerably. There was also no simple and clear relationship between the inhibition of fungal growth and the TPOC concentrations in leachate (Figure 24 The inhibition values were very low at TPOC concentrations below 5 mg/L. The values were considerably higher above this concentration, with high variability observed. In 2001, the TPOC concentrations in the leachates ranged between 3.0-4.2 mg/L and the inhibition was low (≤ 10 %).

During the project, attempts were made to use other fungal species and strains for evaluation of the inhibition of fungal growth by TPOCs in leachate. First of all, strains of ***Trichophyton rubrum***, known to be especially sensitive to the products of hydrocarbon oxidation were evaluated for this purpose. However, this is still an on-going study and its results will be presented in one of the future monthly progress reports.

4.6. Climatic data

The course of soil temperature and moisture in the considered time period depended on the run of insolation determined by the length of day and sun elevation, as well as on weather conditions, especially cloudiness and atmospheric precipitation. The distribution of soil moisture and T_G values also is the function of soil thermal properties, which depend on the kind of soil, porosity and soil ability to cumulate the moisture. The thermal conditions relate to the variation of moisture content in soil. Heating the same volume of soil by 1 ^{o}C needs to convey so many times more calories of heat the more water (in grams) contained in the given soil (Scherban, 1968). In addition, increasing soil moisture causes increase of heat capacity and conductivity and, then, soil heat accumulation and reduction of temperature gradient. It should be emphasized that increasing soil moisture abates the soil albedo (increasing solar energy absorbed by soil) and, simultaneously, increases water evaporation abating soil temperature.

Table 19 presents selected characteristics of climatic conditions in the examined time period. Except for May (V), frequent and high sums of precipitation characterized warm months (IV-IX). Particularly low air temperatures were observed in June (VI) and September (IX). As a consequence, warm months, except for May ($WW_{œr}$ = -1.4 mm, $\sum WW$ = -44.0 mm) displayed the excess of moisture (on average) in the soil profile (positive WW-index values) in relation to the dryness air power. The highest excess of moisture was noticed in July ($WW_{œr}$ = 5.6 mm, $\sum WW$ = 172.4 mm). The months with

the lowest (V) and highest (VII) soil water content were accompanied by the lowest and highest amplitudes of WW index (ΔWW amounts respectively 5.4 mm and 21.7 mm). This agrees with the finding of Wasek (1980), who correlated well the amount of water accumulated in the soil profile up to 50 cm of depth with some indices similar to the WW index. The extreme WW-index values were 18.6 mm (26.VII.2001) and -4.4 mm (17.V.2001).

The course of soil temperature (Table 20 and 21) strongly depended on insolation and the annual run of heat accumulation. This was distinctly observed when compared to the mean soil temperatures and WW-index values in May and August. The months with the lowest soil water content had the mean WW values -1.4 and 0.5 mm, respectively, while the mean T_n values were 19.2 and 25.2 ^oC at 5 cm of depth. The mean T_s values were 15.6 and 20.9 ^oC (highest monthly temperature), respectively. However, high soil moisture in June ($WW_{œr}$ = 3.9 mm) was accompanied by the lower (by 0.3 ^oC) noon temperature at 5 cm of depth as the effect of heat losses from water evaporation (lower than in May by 0.4 ^oC). Simultaneously, diminishing the heat re-emission in the nighttime (in relation to the value from May) caused higher T_s values. The values of noon or daily mean temperature in the soil profile (between 5 and 50 cm of depth) generally refer to WW-index values. The highest differences in T_n (also T_s) were observed in May and August (5.9 and 5.6 ^oC). The smallest difference was in September (0.7 ^oC), due to the effect of lower insolation. The negative values of radiation balance in the period of January-March 2001 reflected the negative temperature differences.

In general, warm winter months (January and February) and cold (except for August) and humid (except for May) vegetation season characterized the period of January-September 2001. Except for May, the excess of water in soil (positive mean WW-index values) was observed during the whole vegetation season. The highest mean and extreme soil temperature (30.5 ^oC at 5 cm of depth) was observed in August. It was accompanied by the considerable temperature gradients (≈ 1.0^o/10 cm, ΔT_n = 5.6 ^oC) between 5 and 50 cm of soil depth. Only the highest temperature gradients amounting ≈ 1.3^o/10 cm (ΔT_n = 5.9 ^oC) were observed in May, due to the soil dryness ($WW_{œr}$ = -1.4 mm). Soil temperatures were (on average) positive at the depths of 5 to 50 cm in winter months, frequently because of snow cover. It mainly refers to January 2001.

4.7. Data from the non-engineered biopile

The construction of the non-engineered biopile was completed at the beginning of November 2001. The first (preliminary) sampling campaign at this biopile was organized by the end of this month. Results of physico-chemical and microbiological analyses will be available in December 2001 and presented, together with a description of the technology, in a monthly progress report.

5. Discussion

The bioremediation process at the engineered biopile was generally divided into four major stages. The first stage underwent through the 7th month of bioremediation and

was characterized by the considerable decrease of petroleum contaminants in soil. In the passive section and shallow layer of the biopile, the first stage lasted longer (through the 9th month of bioremediation). However, much higher removals of contaminants were observed in the active section and deep layer of the biopile. The considerable decrease of contaminants was associated with high dehydrogenase activity. These phenomena can be explained with intensive aeration, mineral fertilization and high initial concentrations of hydrocarbon contaminants in the active section/deep layer of the biopile. However, a distinct increase of contaminants was noticed between the 7th and 21st months of bioremediation. This long time period was the second stage of bioremediation. The increase could better be observed in the passive section and shallow layer than in the active section and deep layer of the biopile. The microbial activity during the second stage of bioremediation was rather low. The second considerable decrease of hydrocarbon contaminants was noticed between the 21st and 24th months of bioremediation. This short time period was the third stage of the process. The decrease of contaminants can be explained with the first treatment of the biopile with the surfactant, Rokafenol N8. It is widely accepted that both natural and synthetic surfactants increase the availability of hydrocarbons (by desorption, solubilization and emulsification) to the microorganisms able to degrade the contaminants in soil (Karickhoff ***et al***., 1979; Cooper & Zajic, 1980; Rao ***et al.***, 1993; Mihelcic ***et al.***, 1995). However, it is difficult to explain no increase of microbial activity during the third biopile bioremediation stage.

The fourth bioremediation stage began in the 24th month and lasted until present so that included 2001 MNA activities. The fourth stage was generally characterized by high fluctuations of hydrocarbon concentrations and microbial activity. One of the major findings was that the concentrations of polar compounds were much lower in 2001 than previous concentrations. Subsequently, a considerable decrease of contaminants was observed between February and July (between the 39th and 44th months of bioremediation). This decrease was associated with extremely high microbial activity so that the dehydrogenase activity in July was the highest during the whole bioremediation project (1997-2001). However, the concentrations of contaminants markedly increased and the microbial activity decreased in October, which were especially well observed in the former active section and deep layer of the biopile. No clear seasonal changes, but clearly stabilized conditions, were noticed in the former passive section of the biopile. It is difficult to explain the above-mentioned phenomena. It is believed that intensive NPK fertilization (addition of mineral nitrogen, in particular), along with the addition of Rokafenol N8 to the soil, caused the increase of hydrocarbon availability to microorganisms, high microbial activity and high removal of hydrocarbons, including polar compounds, from the soil in the former active section of the biopile. This underwent under rather unfavorable whether conditions, which were relatively low temperatures and high water excess (positive WW-index values) in the soil profile in summer. However, the temperature decreased and high soil moisture maintained in autumn, which resulted in the drastic decrease of microbial activity and the increase of hydrocarbon concentrations in October. A possible leakage and movement of contaminants from the non-engineered biopile (during its construction) to the former active section of the engineered biopile also could add to this increase.

The PAH changes were less considerable than the changes of aliphatic hydrocarbons during bioremediation at the biopile. This can be explained with higher resistance of PAHs to biodegradation. It is noteworthy, however, that the general PAH mass loss was higher than the general TPH/TPOC mass loss during the whole bioremediation period. The course of PAH changes during bioremediation also can be divided into several stages. The PAH removal was the most efficient through the 18th month of bioremediation. The PAH means were "stable" between the 18th and 24th months and increased in the 39th month of the process (February 2001). In 2001, a decrease of PAHs was observed to concentrations comparable with the concentrations from the PAH-stabilized bioremediation stage. The fluctuations in PAH concentrations probably resulted from the heterogeneity of soil at the engineered biopile. When the concentrations of contaminants in soil are low, it is difficult to distinguish the effects of biodegradation process and heterogeneity of the soil examined.

In the Microtox® studies, only ethanol extracts of two mixed soil samples 5 and 6 displayed toxicity. This finding confirmed the conclusion of Munkittrick & Power (1991) that the Microtox® Test System is more sensitive to organic extracts than to water extracts of soil. However, results from both types of extracts should be taken for ecotoxicity interpretation. The analysis of physico-chemical results showed low concentration of toxic heavy metals in sample 5 and 6. However, sample 5 had the highest concentration of hydrocarbon contaminants, including PAHs. Therefore, these ethanol-soluble contaminants appear to be the major toxic factors in this sample. However, no essential differences were observed between the concentrations of hydrocarbons in sample 6 and in the other samples (excluding sample 5). Thus, at the present stage of the studies, toxic factors in sample 6 remain unknown. The conclusion from the Microtox® studies is that subsection II of the biopile (Figure 1) is still dangerous from an ecotoxicological point of view. It is not surprising, because the PAH concentrations have been the highest in this subsection since the beginning of the bioremediation project (IETU, 1999). To complete the ecotoxicity data, it is suggested to examine mixed samples from the biopile with the Ames mutagenecity/toxicity test or other tests, which are especially sensitive to mutagenic compounds.

The inhibition of ***Trichophyton ajelloi*** R66 growth by leachate was very high through the 6th month of bioremediation and drastically decreased between the 6th and 9th month of the process. During the following months, the inhibition values were low with, however, distinct fluctuations observed. It is noteworthy that the bioremediation stages established on the basis of fungal growth inhibition by leachate generally fit with the stages based upon the TPH/TPOC changes in soil. This conclusion mainly concerns the first and second stage of bioremediation in soil studies. The low inhibition of fungal growth was associated with TPOC concentrations in leachate below 5 mg/L. In 2001, the TPOC concentrations in leachate ranged between 3.0-4.2 mg/L. It was not surprising, therefore, that the inhibition of fungal growth was low (≤10 %). The studies are still on going. The results appear to confirm the usefulness of the fungal test in bioremediation studies but mainly for assessment of initial bioremediation stages, during which concentrations of toxic polar compounds (POLARs) in leachate are high. The study indicated the need for a fast and easy fungal test for leachate, employing strains

with sensitivity to hydrocarbon contaminants higher than that of ***Trichophyton ajelloi***. It appears that strains of other dermatophyte species, ***Trichophyton rubrum*** in particular, may meet this requirement. It is noteworthy that pathogenic fungi, ***Trichophyton rubrum*** together with ***Candida albicans***, have already been used for ecotoxicity bioassay (Rychta ***et al.***, 2001). Possible studies with ***Trichophyton rubrum*** strains should be developed for future applications.

Keratinolytic and keratinophilic fungi occurred in soil at the engineered biopile with relatively high frequencies. This can be explained with the fungal ability to degrade aliphatic hydrocarbons in the presence of proteins (Garg ***et al.***, 1985; Ulfig ***et al.***, 2000; Przystas ***et al.***, 2001). The studies emphasized the ecotoxicological aspect of the bioremediation process at the biopile. This aspect was, however, directly associated with changes in fungal biodiversity during bioremediation. The long-term data obtained with the traditional Vanbreuseghem's method (with incubation at room temperature - ± 23 ^{o}C) showed relationships between the TPOC concentrations and fungal frequency, the frequency of ***Trichophyton ajelloi,*** in particular. The cumulative frequency plots suggested that polar compounds (POLAR) together with PAHs and dehydrogenase activity (TTC) chiefly inhibited the growth of this fungus in soil under bioremediation . Non-polar aliphatic compounds appeared to play a minor role in the inhibition. Similarly to the data obtained in leachate studies, however, ***Trichophyton ajelloi*** also displayed the moderate sensitivity to hydrocarbons in soil. TPOCs below 15 g/kg d.w. did not affect, or affected to a minimal degree (high variability observed), the growth of the fungus in soil. The data confirmed the usefulness of the method in bioremediation studies. However, there is a need to identify fungal species with higher sensitivity to hydrocarbons in soil or more sensitive modification of the method for ecotoxicity/biodiversity bioremediation assessment. The 4-temperature method provides more information than the traditional method on changes in the fungal community in soil under bioremediation. It appears that this method, with the hydrocarbon-sensitive species, ***Chrysosporium keratinophilum,*** may meet the goal.

6. Project Benefits

The project continued the bioremediation studies at the refinery. Its primary goal was to compare long- and short-term data from monitoring the bioremediation process at the engineered biopile. Based on results obtained, the bioremediation period (1997-2001) was divided into four stages. The stages considerably differed in aliphatic hydrocarbon/PAH removal rates, microbial activity and composition. The fourth stage included activities conducted in 2001. Bioremediation in 2001 were carried out with natural aeration, application of mineral NPK fertilizers and the surfactant, Rokafenol N8, to the soil to stimulate the removal process of recalcitrant hydrocarbons from the biopile. The application of these methods with employing indigenous microorganisms is often referred to as monitored natural attenuation (MNA). High hydrocarbon removals (up to 77.71%) confirmed the usefulness of the biopile technology for cost-effective soil clean up purposes. Subsequently, characterization of the bioremediation stages is of benefit to making plans for future soil clean up projects.

Successful bioremediation results obtained by the DOE/IETU staff inspired the refinery management, in close cooperation with IETU specialists, to design and build another biopile to remediate soil mixed with petroleum hydrocarbon waste in the second lagoon at the refinery. The new biopile uses a simple and cost-effective ***in situ*** bioremediation technology, with a drainage system and dolomite layer covering the contaminated soil, but its bioremediation process is presumably carried out under very passive (almost anaerobic) conditions. In contrast to the DOE/IETU engineered biopile, the new biopile was, therefore, called the non-engineered biopile. Construction of the new biopile was completed at the beginning of November 2001. The first sampling campaign was performed by IETU at the end of December. It is intended to compare MNA at the engineered and non-engineered biopiles next year. This also should be of benefit to making plans for future soil clean up projects.

The project continued several novel approaches to MNA at the engineered biopile. The novelties chiefly concerned the assessment of ecotoxicity and biodiversity during bioremediation. An essential novelty was the evaluation of soil and leachate toxicity with the Microtox® Test System. The Microtox® studies showed that, due to the presence of toxic compounds, mainly PAHs, the former active section of the engineered biopile was still dangerous from an ecotoxicological point of view. The conclusion was that soil samples from the biopile also should be examined with the Ames mutagenecity/toxicity test or/and other tests with high sensitivity to mutagenic compounds. Results of these tests will complete the ecotoxicity assessment of the biopile soil after a long-term bioremediation. Several methods using microscopic fungi also were developed for assessment of ecotoxicity and biodiversity during bioremediation.

The IETU team, in close cooperation with specialists from WSRC, will develop the plans for further physico-chemical and microbiological analyses at the biopiles. In addition, common activities between the IETU and WSRC will continue the characterization of the biopile microbial community. The goal of these common activities is not only to evaluate the reaction of microbes to contaminants but also determine alternative microbial pathways (co-metabolic biodegradation) under laboratory conditions for the remediation of recalcitrant hydrocarbons.

Petroleum hydrocarbon contamination of terrestrial and aquatic environments is more and more common at urban and industrialized sites throughout the country. A number of DOE sites lists hydrocarbon contamination as an environmental problem in the context of remediation technology needs. Existing technologies typically involve excavation and off-site disposal. These technologies are costly, particularly when the contaminated area is large. Bioremediation is a promising innovative technology that has great potential and gained much interest throughout the DOE complex. The investment in monitoring the biopiles supports both the bioremediation efforts, by providing long-term data from this project, and the growing remedial option known as MNA. Both of these aspects are of benefit to DOE in meeting clean up goals.

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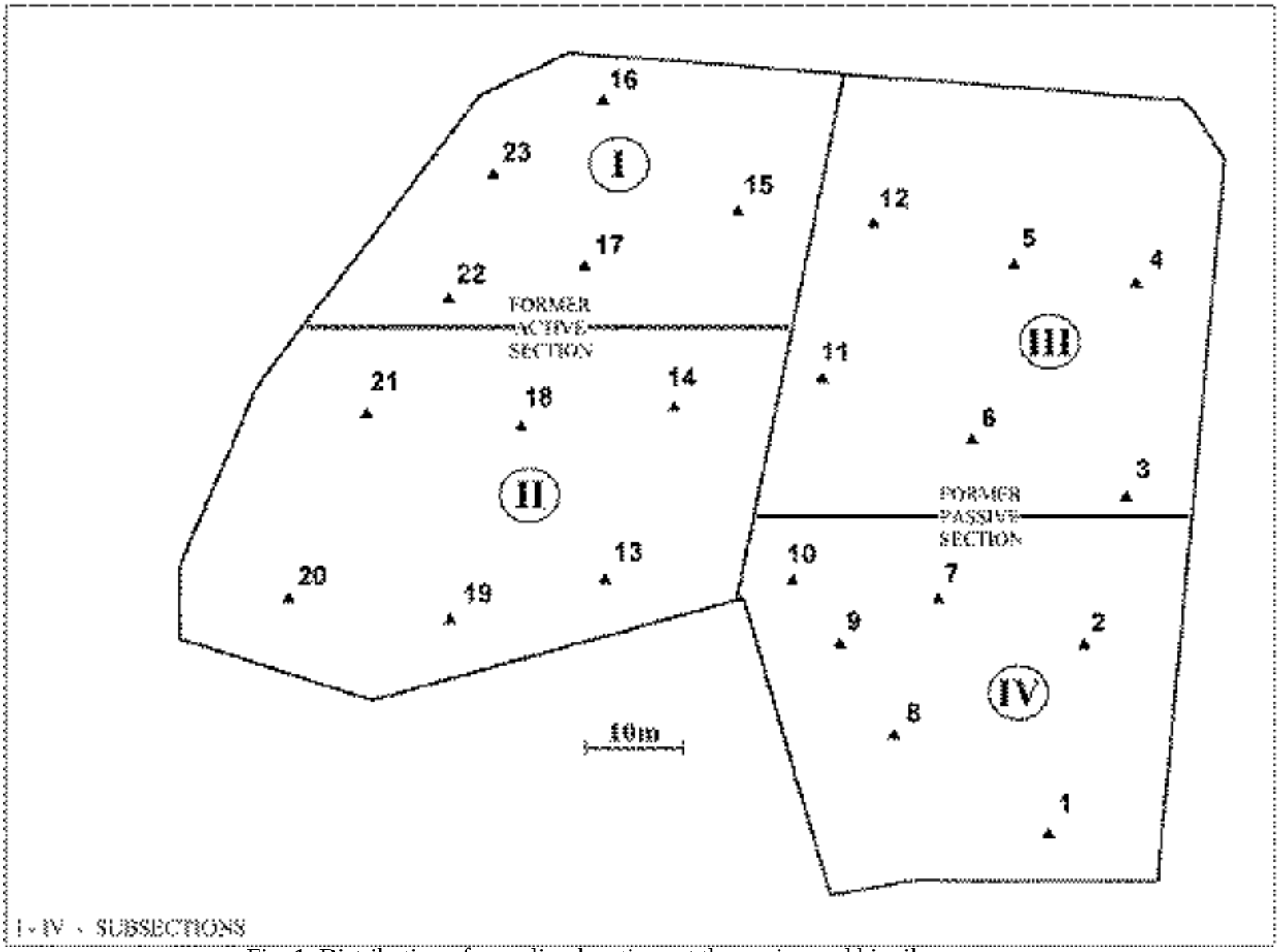

Fig. 1. Distribution of sampling locations at the engineered biopile

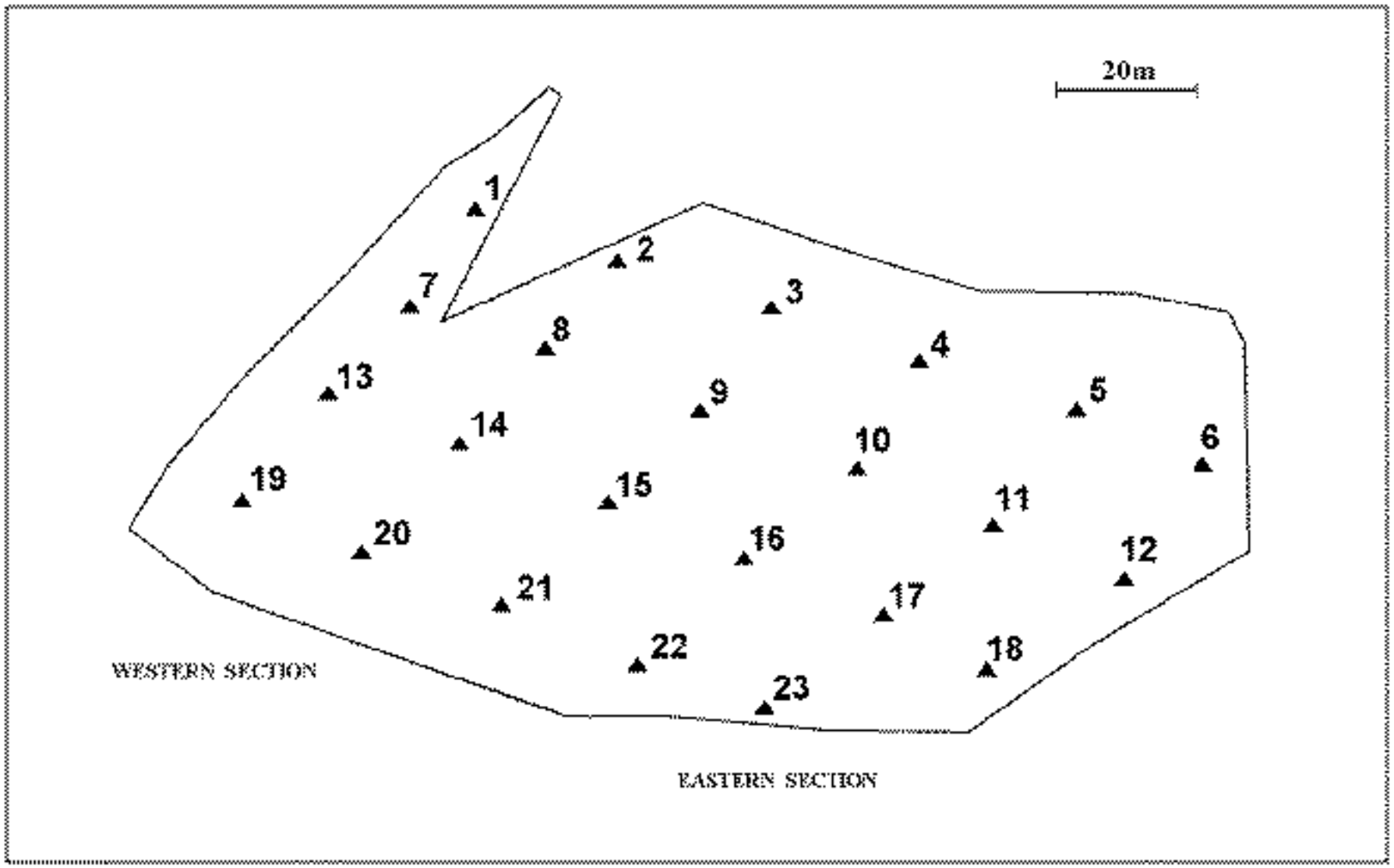

Fig. 2. Distribution of sampling locations at the non-engineered biopile

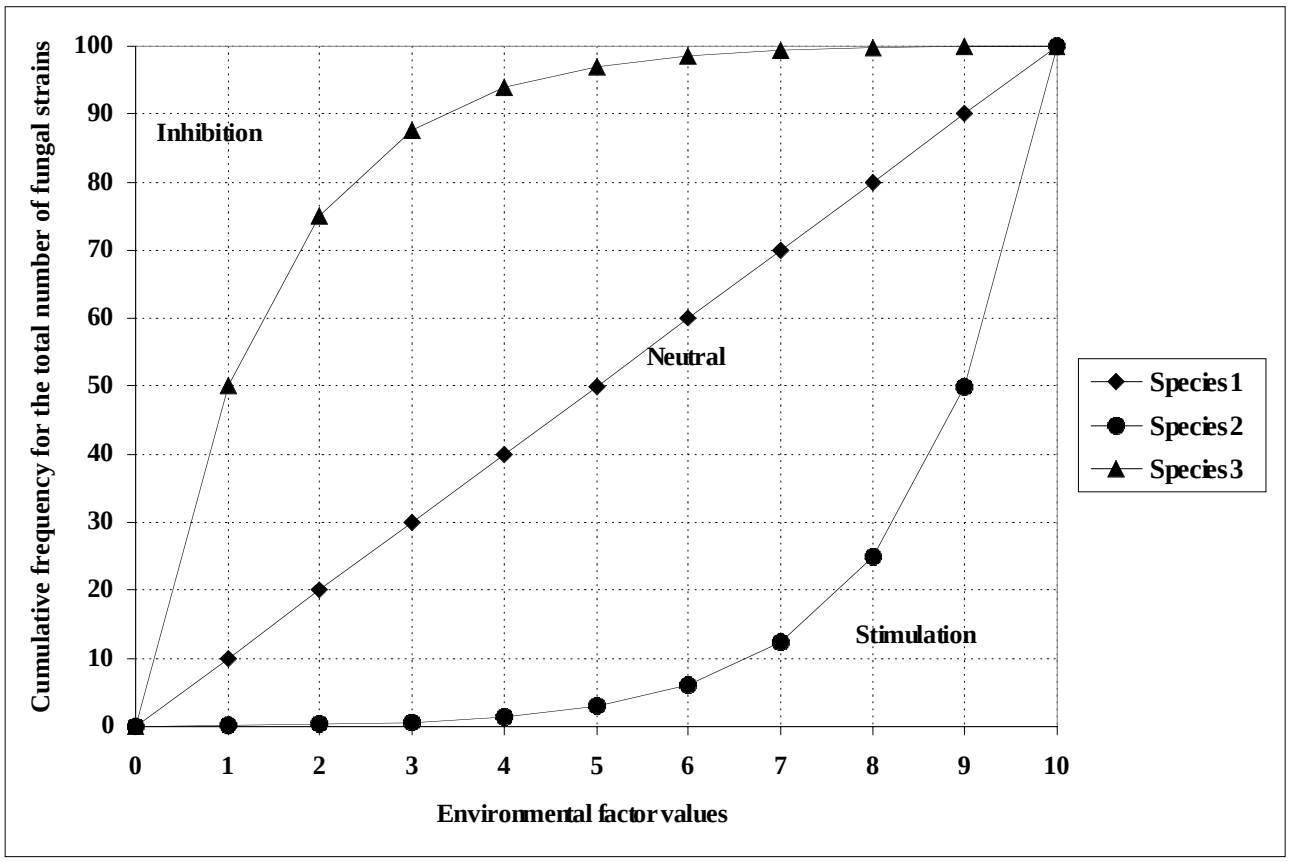

Fig. 3. Relationships between the cumulative frequency for the total number of fungal strains with the values of an environmental factor for three fungal species

Fig. 4. Keratinolytic and keratinophilic fungi growing on biopile samples at different temperatures

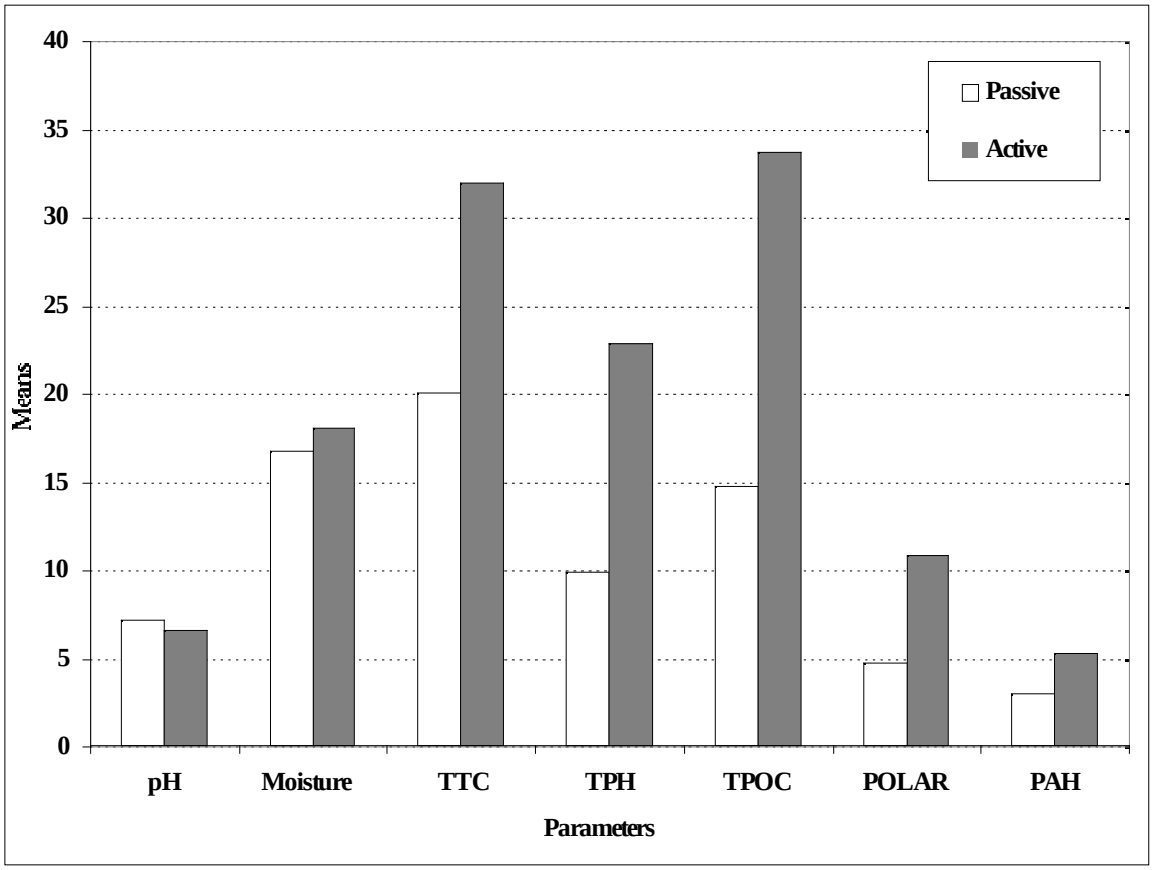

Fig.5. Means for selected physico-chemical parameters for active and passive sections of the engineered biopile

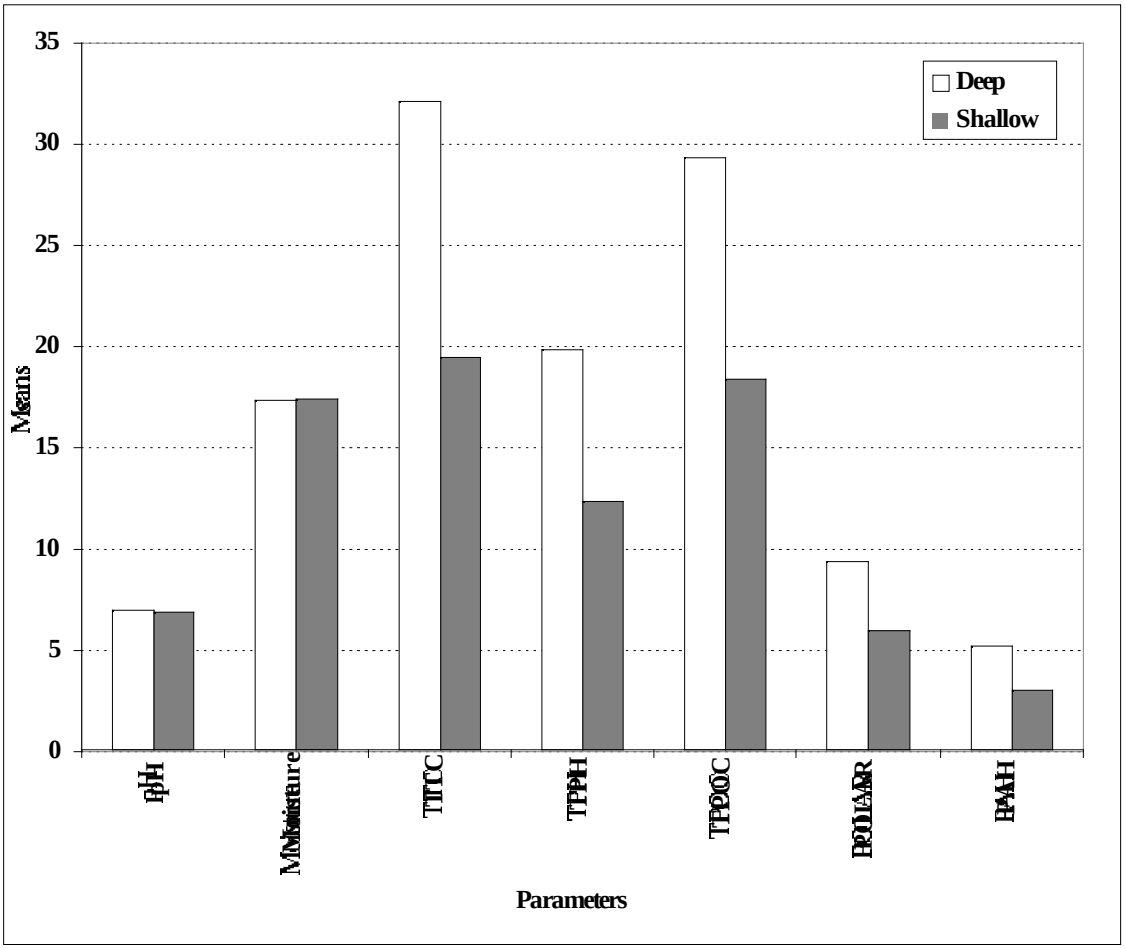

Fig. 6. Means for selected physico-chemical parameters for shallow and deep layers of the engineered biopile

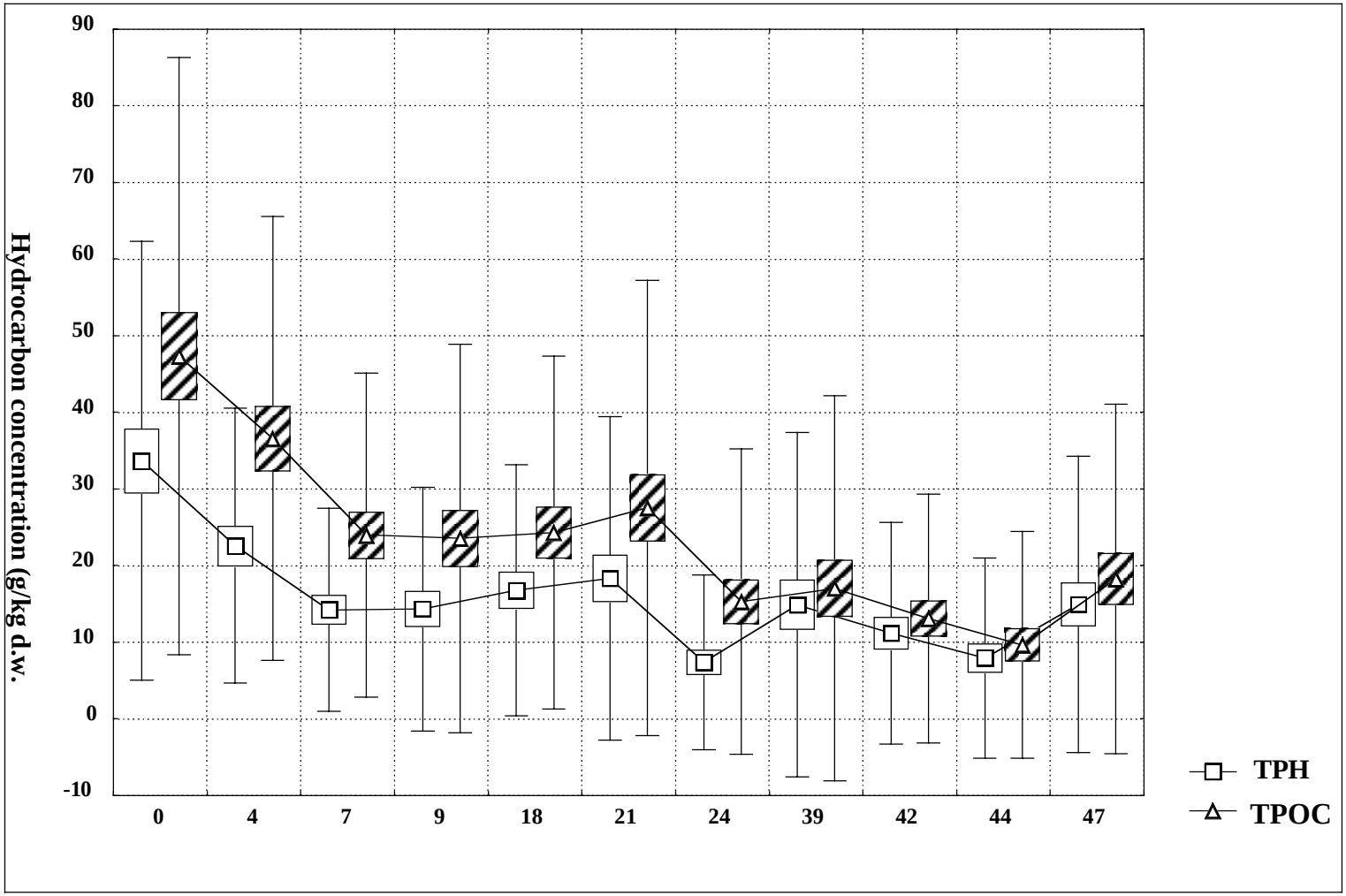

Fig. 7. Changes in soil TPH/TPOC concentrations during bioremediation at the engineered biopile

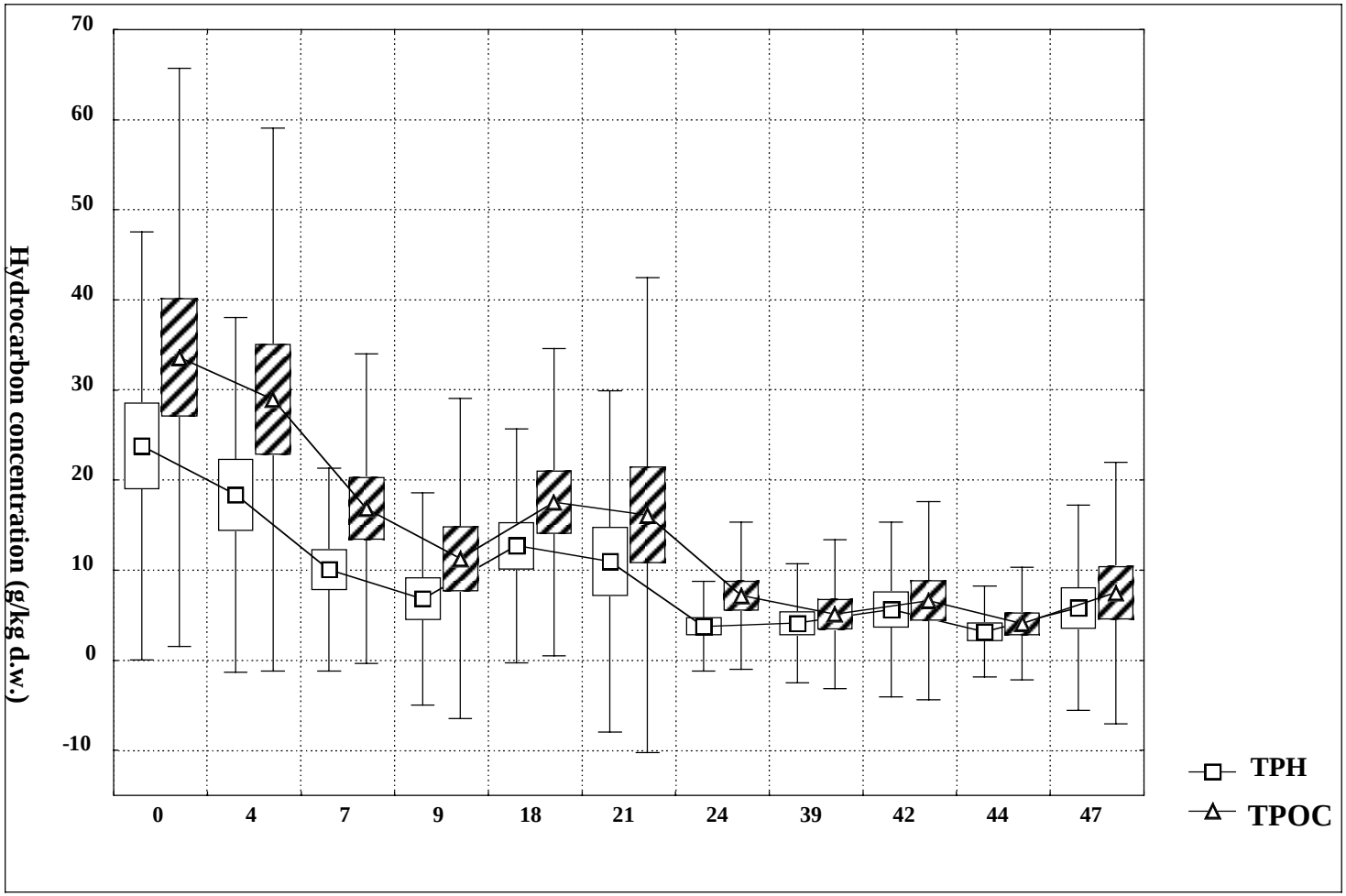

Fig. 8. Changes in soil TPH/TPOC concentrations during bioremediation in the passive section of the engineered biopile

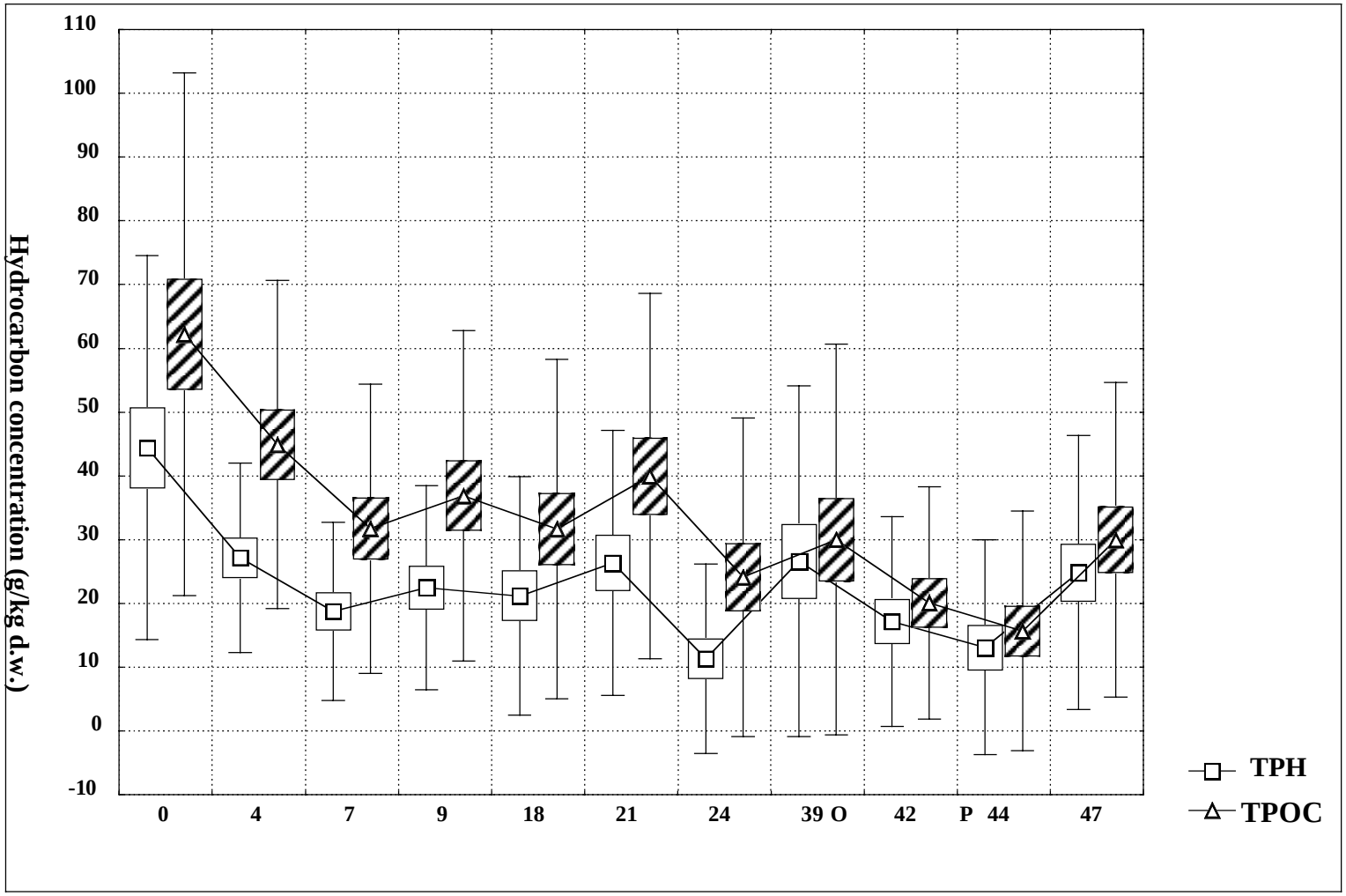

Fig. 9. Changes in soil TPH/TPOC concentrations during bioremediation in the active section of the engineered biopile

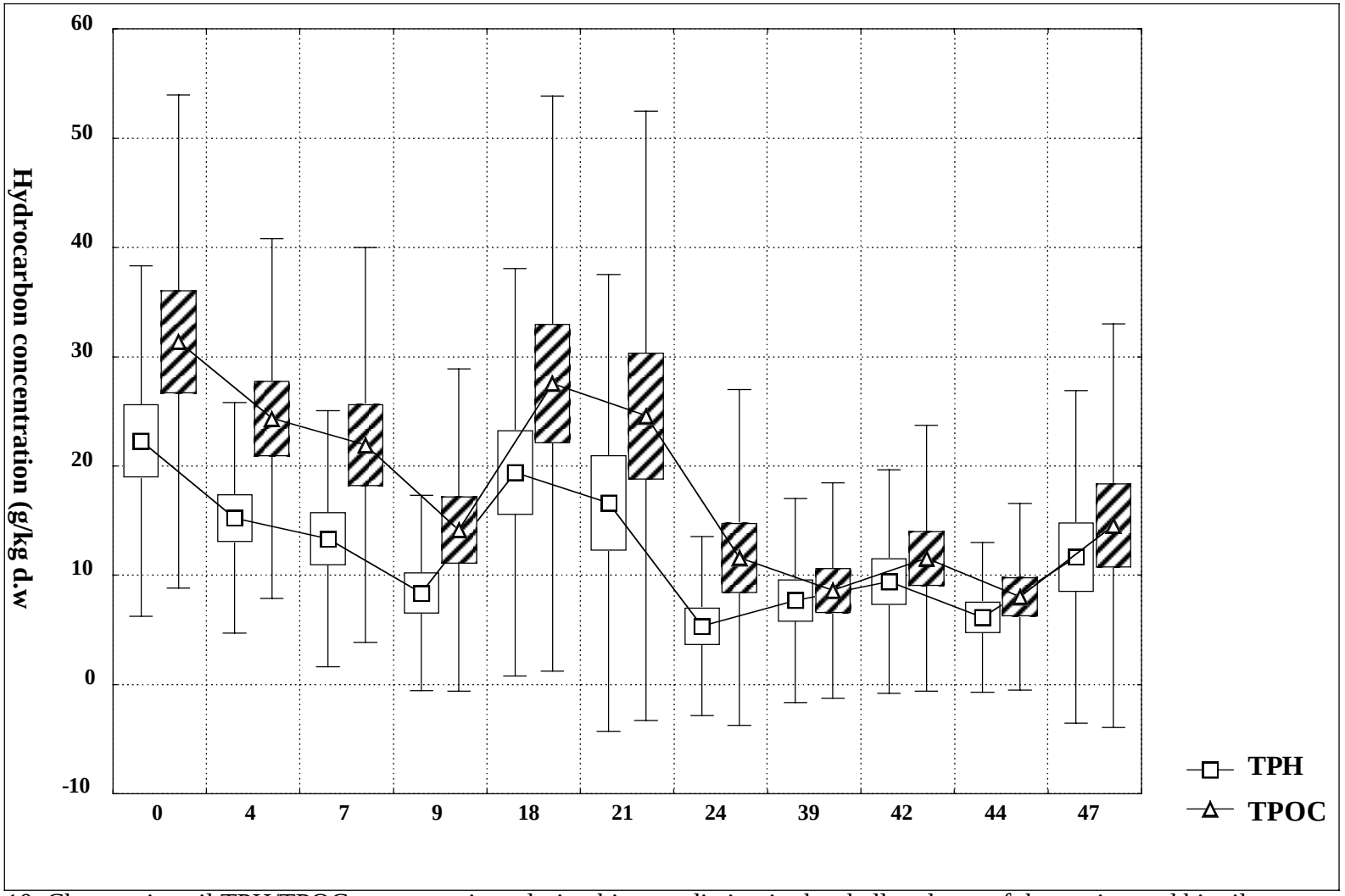

Fig. 10. Changes in soil TPH/TPOC concentrations during bioremediation in the shallow layer of the engineered biopile

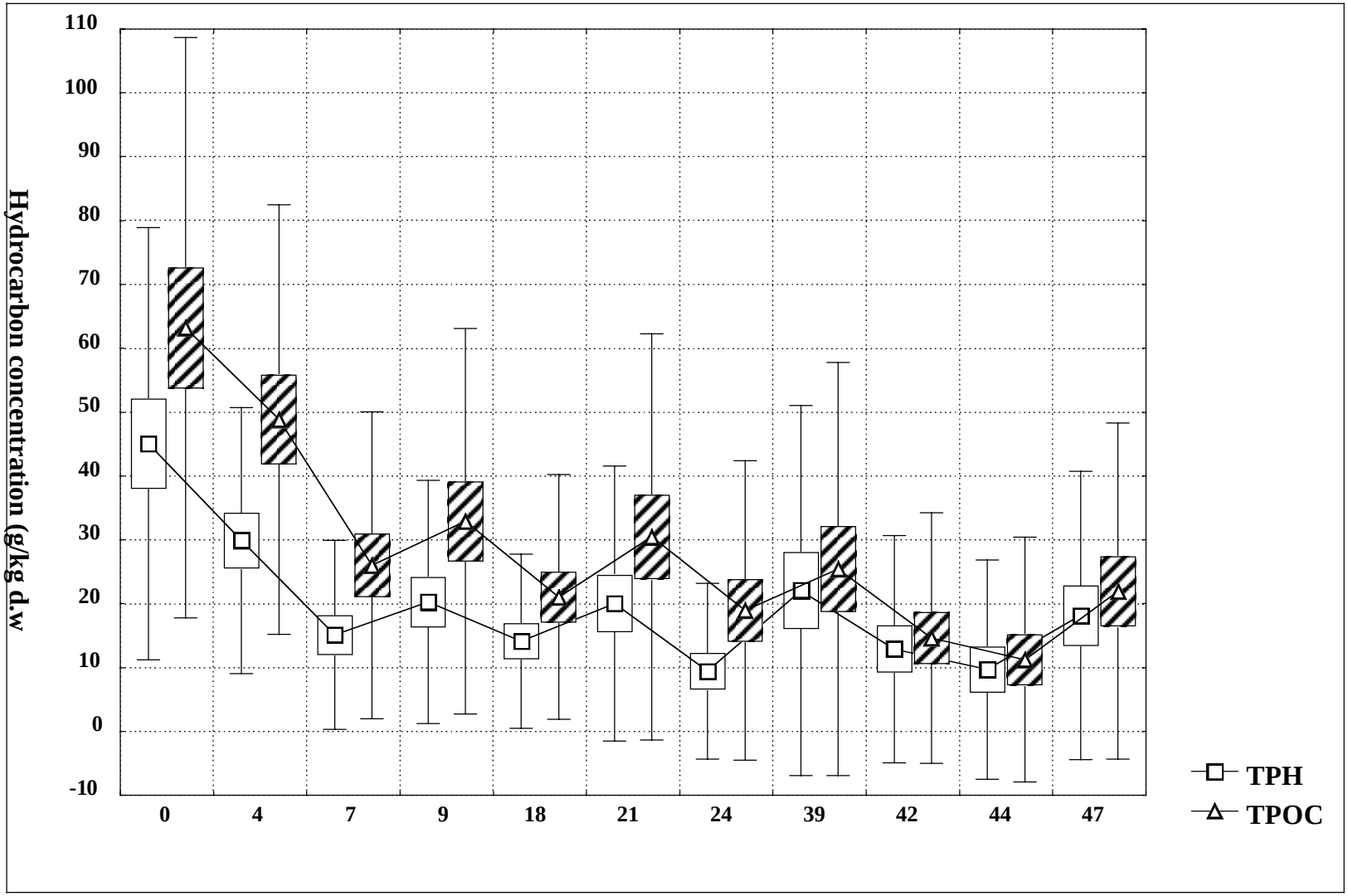

Fig. 11. Changes in soil TPH/TPOC concentrations during bioremediation in the deep layer of the engineered biopile

Fig. 12. Smooth-walled macroconidia of *Trichophyton ajelloi* (x 400)

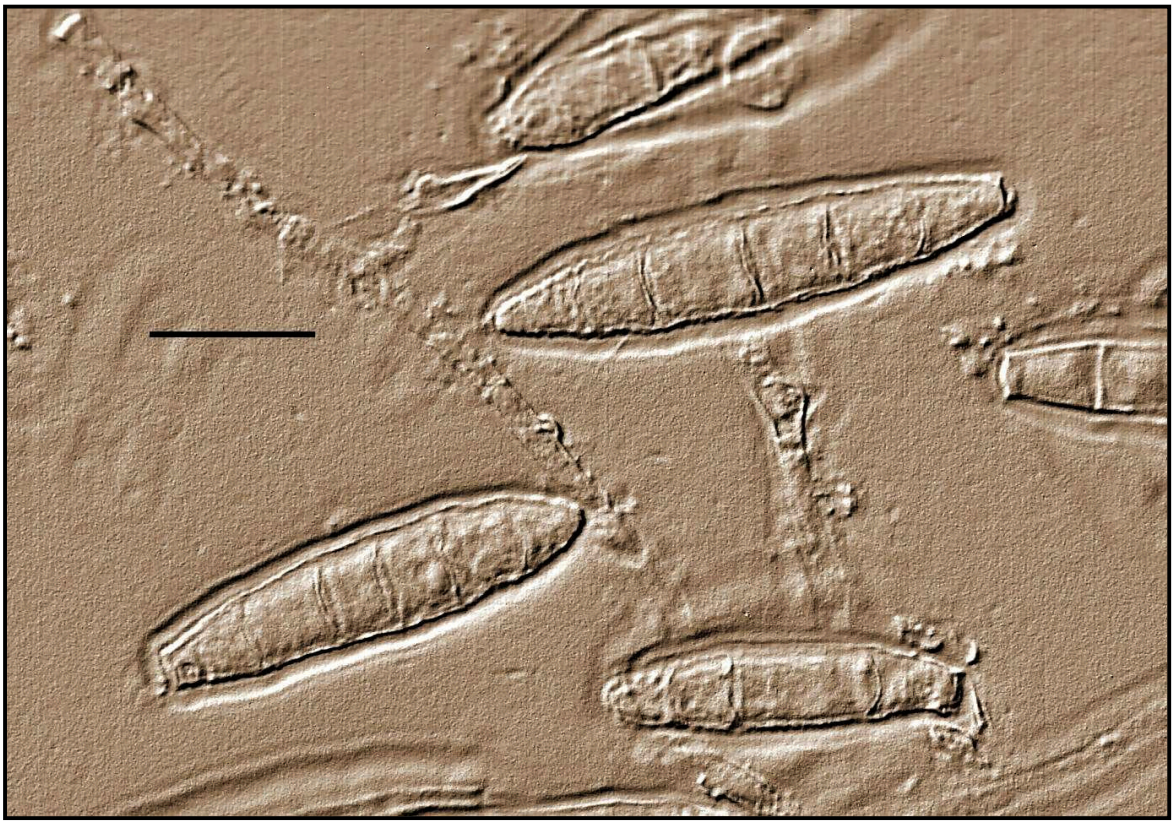

Fig. 13. Macroconidia of *Microsporum gypseum* with echinulate walls (bar – 10 _m) isolated from the engineered biopile

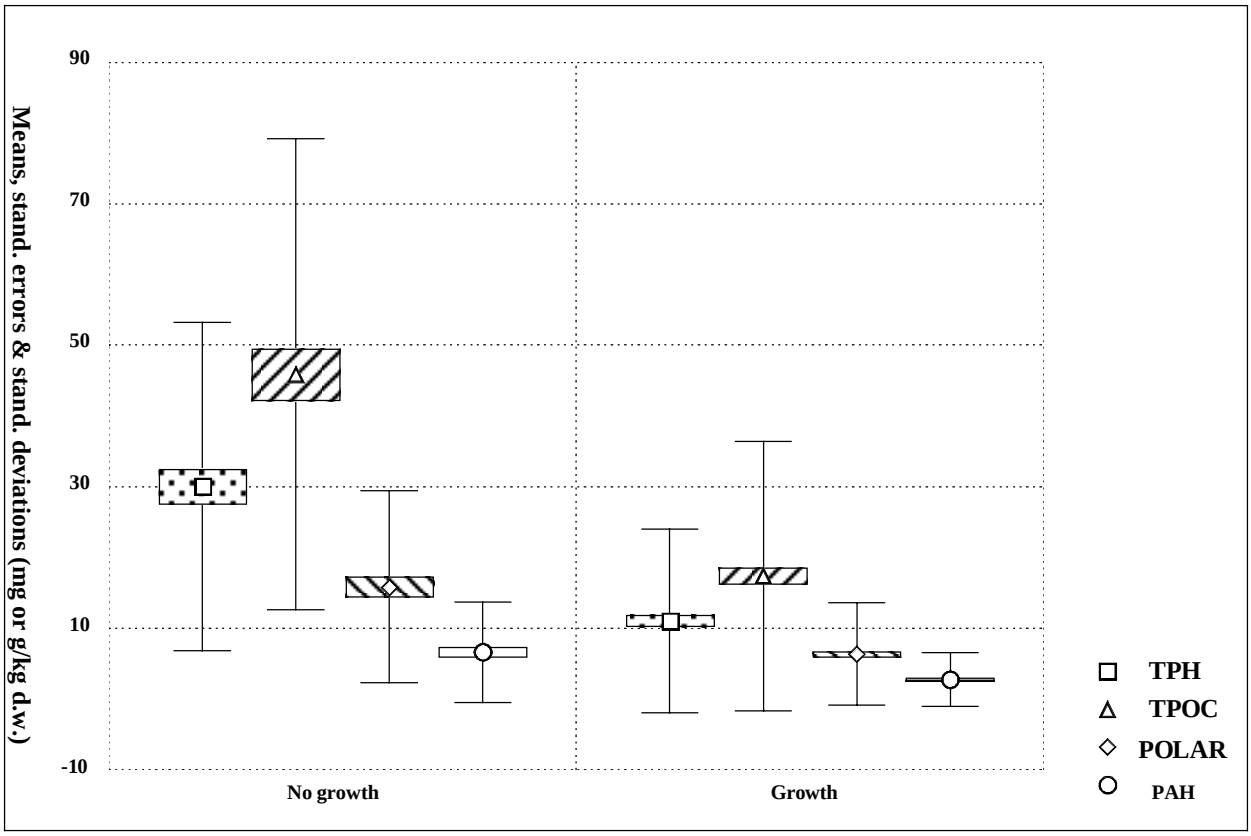

Fig.14. Relations between the concentrations of TPOC and the occurrence of *Trichophyton ajelloi* in soil from the engineered biopile

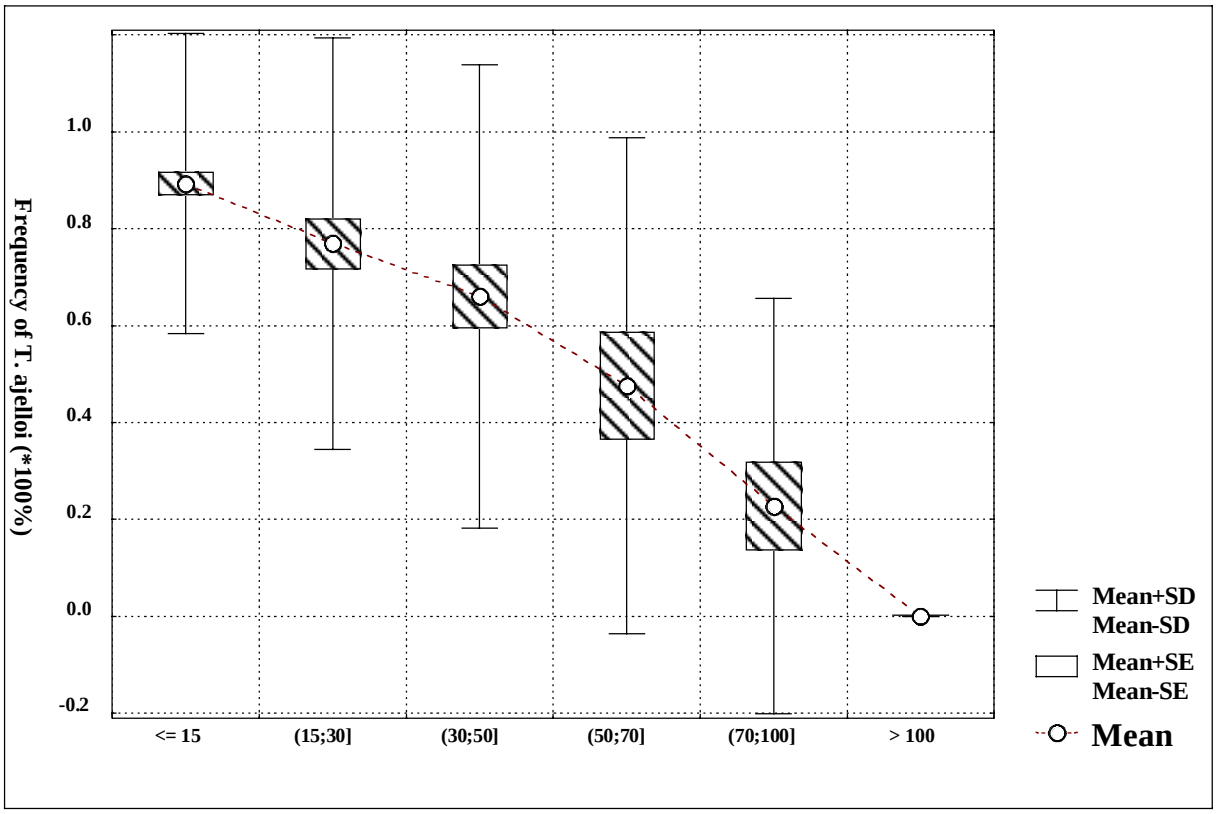

Fig.15. Relation between the frequency of *Trichophyton ajelloi* and the concentrations of TPOC in soil from the engineered biopile

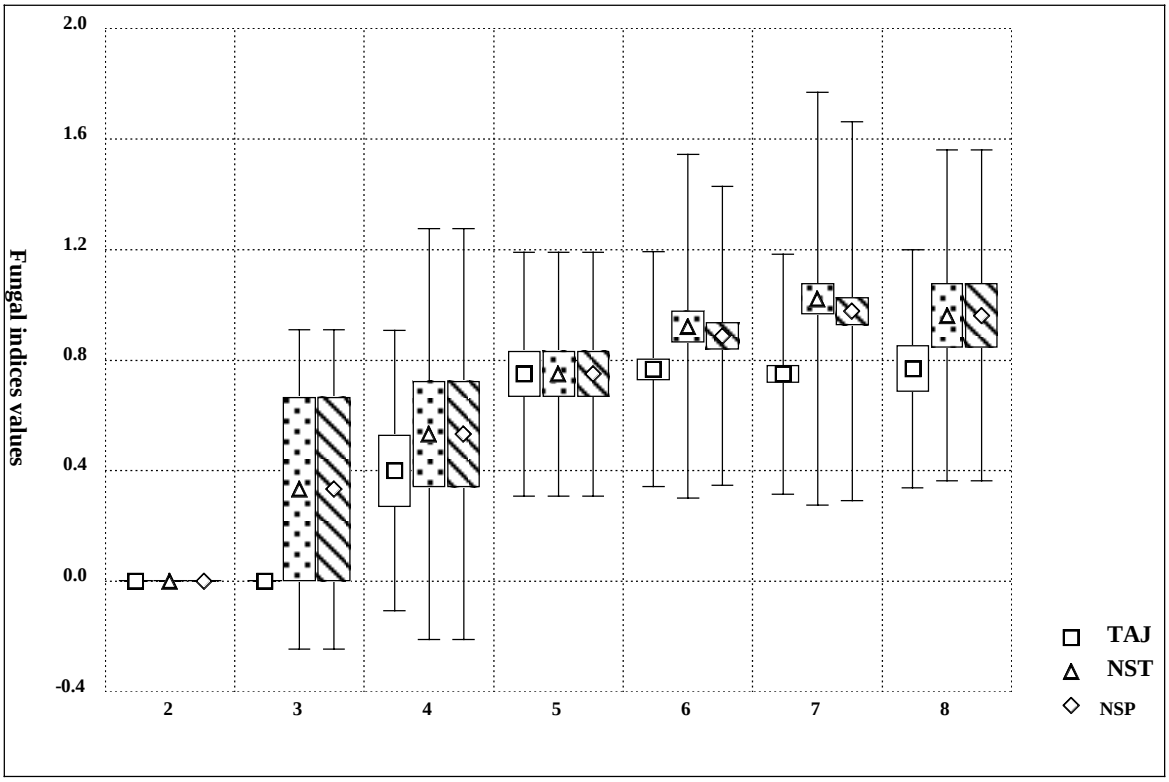

Fig.16. Relation between the occurrence of keratinolytic fungi and pH in soil from the engineered biopile

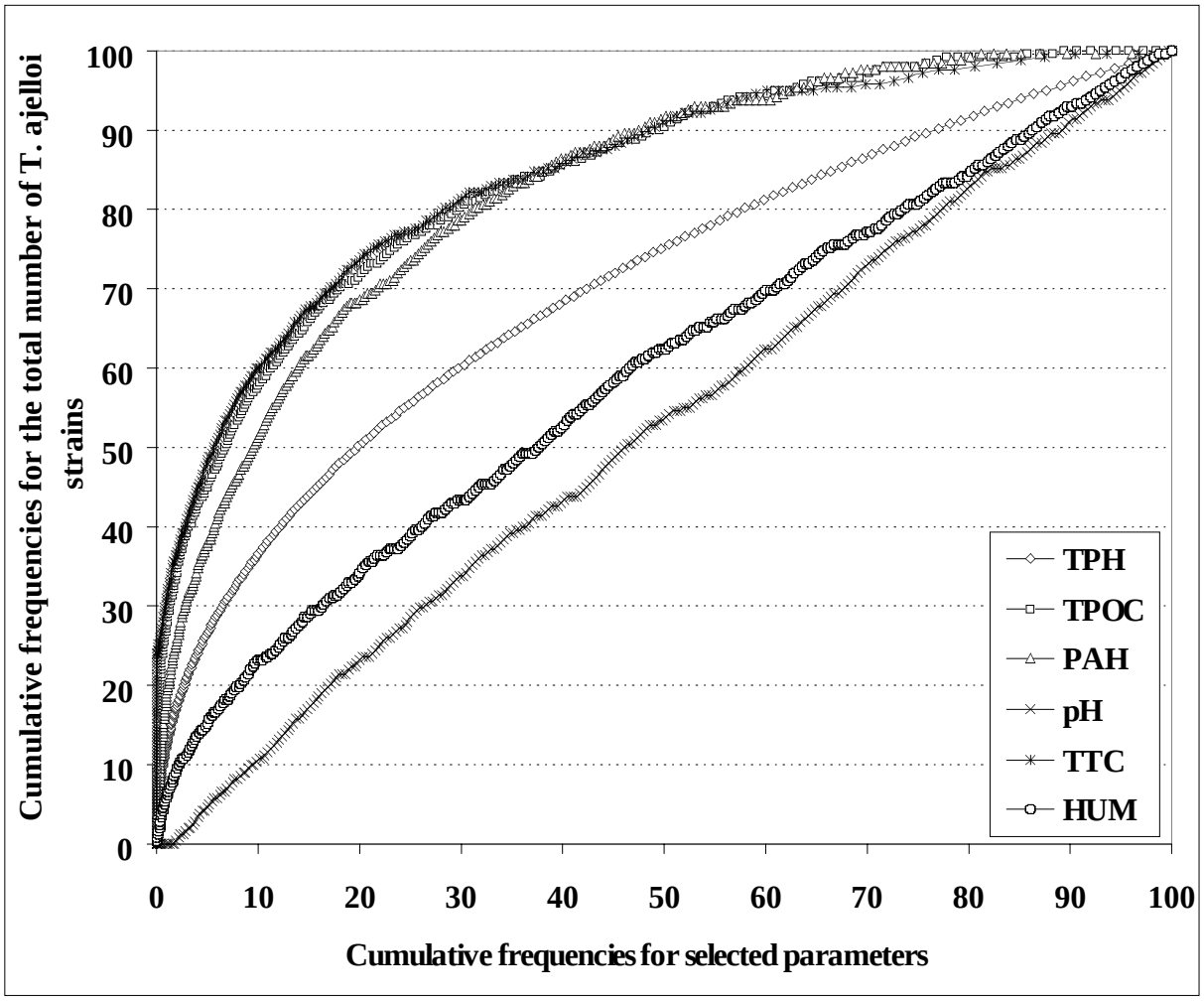

Fig. 17. Relationships between the cumulative frequencies for the total number of *Trichophyton ajelloi* strains and the cumulative frequencies for selected parameters

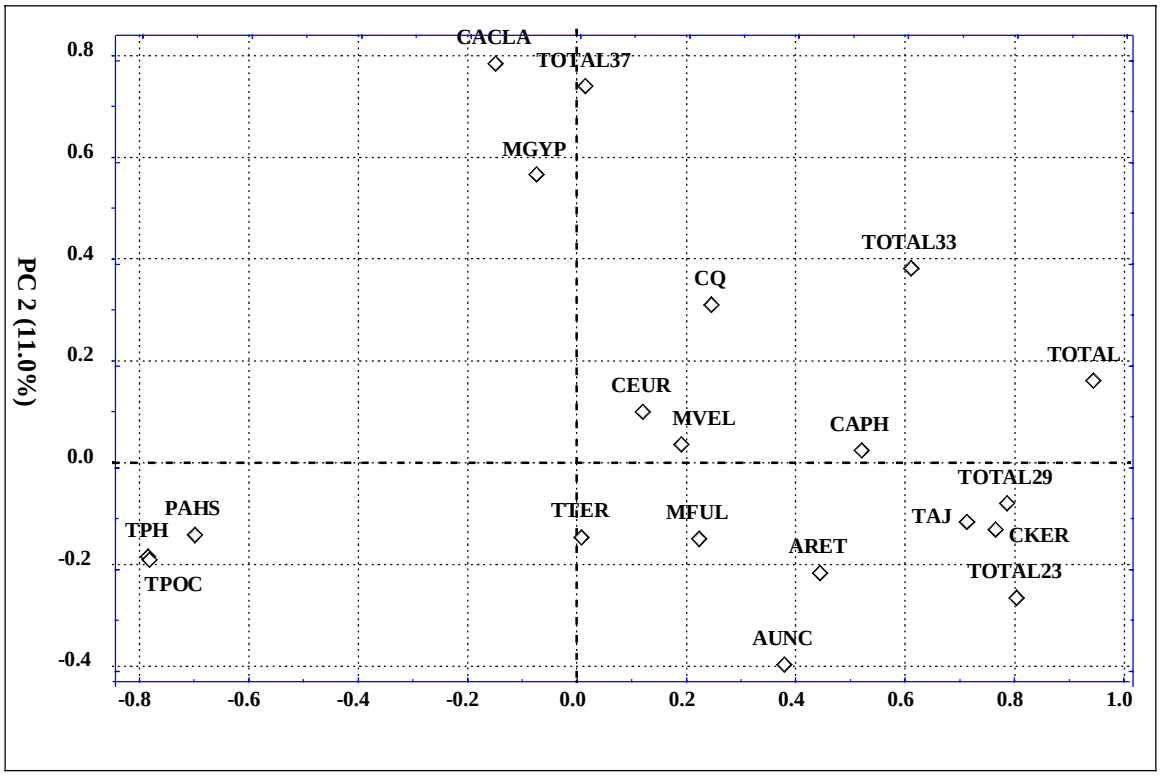

Fig. 18. Relationships between physico-chemical and mycological parameters. PCA analysis for data obtained at four temperatures (23, 29, 33 i 37 ^{o}C) in 2001

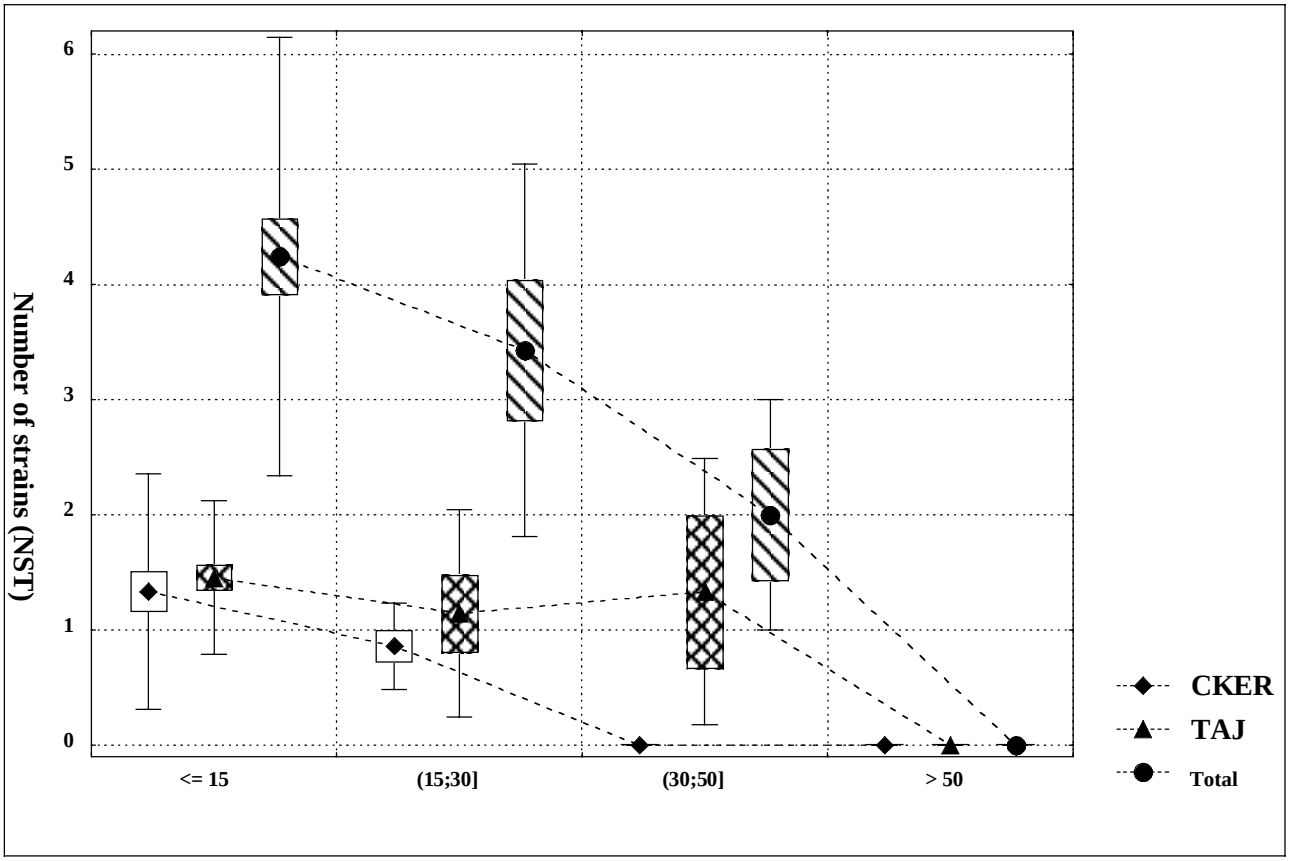

Fig. 19. Relationship between the number of isolated strains (NST) and TPOC for data obtained at the engineered biopile in 2001 at four incubation temperatures (23, 29, 33 and 37 ^{o}C)

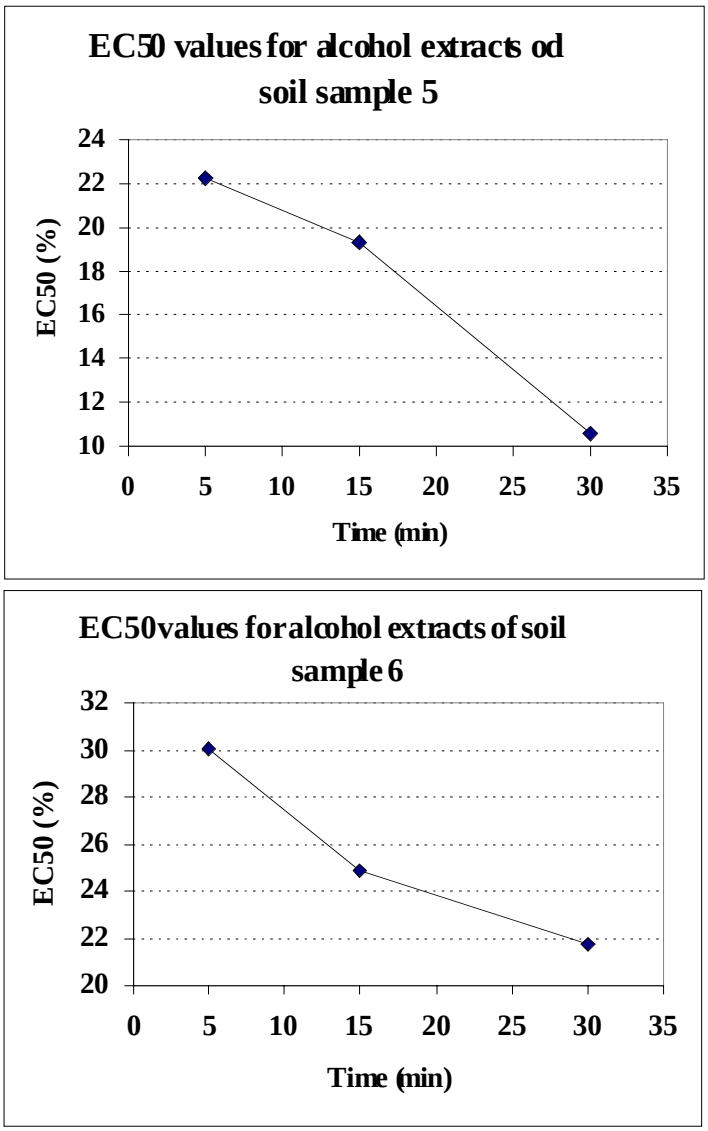

Fig. 20. EC_{50} values for alcohol extracts of mixed soil samples 5 and 6

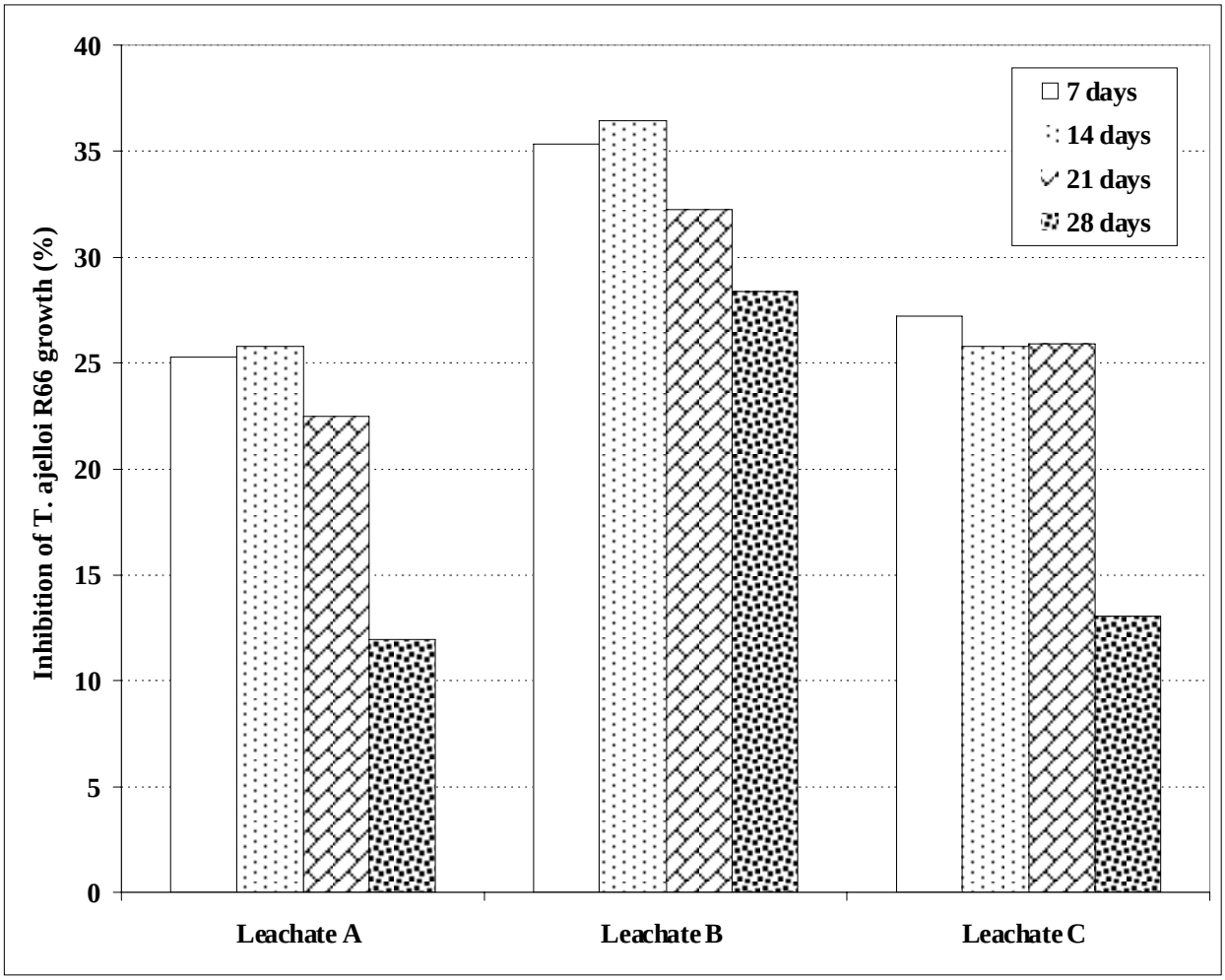

Fig. 21. Relationships between the inhibition of *Trichophyton ajelloi* strain R66 linear growth and the incubation time in days

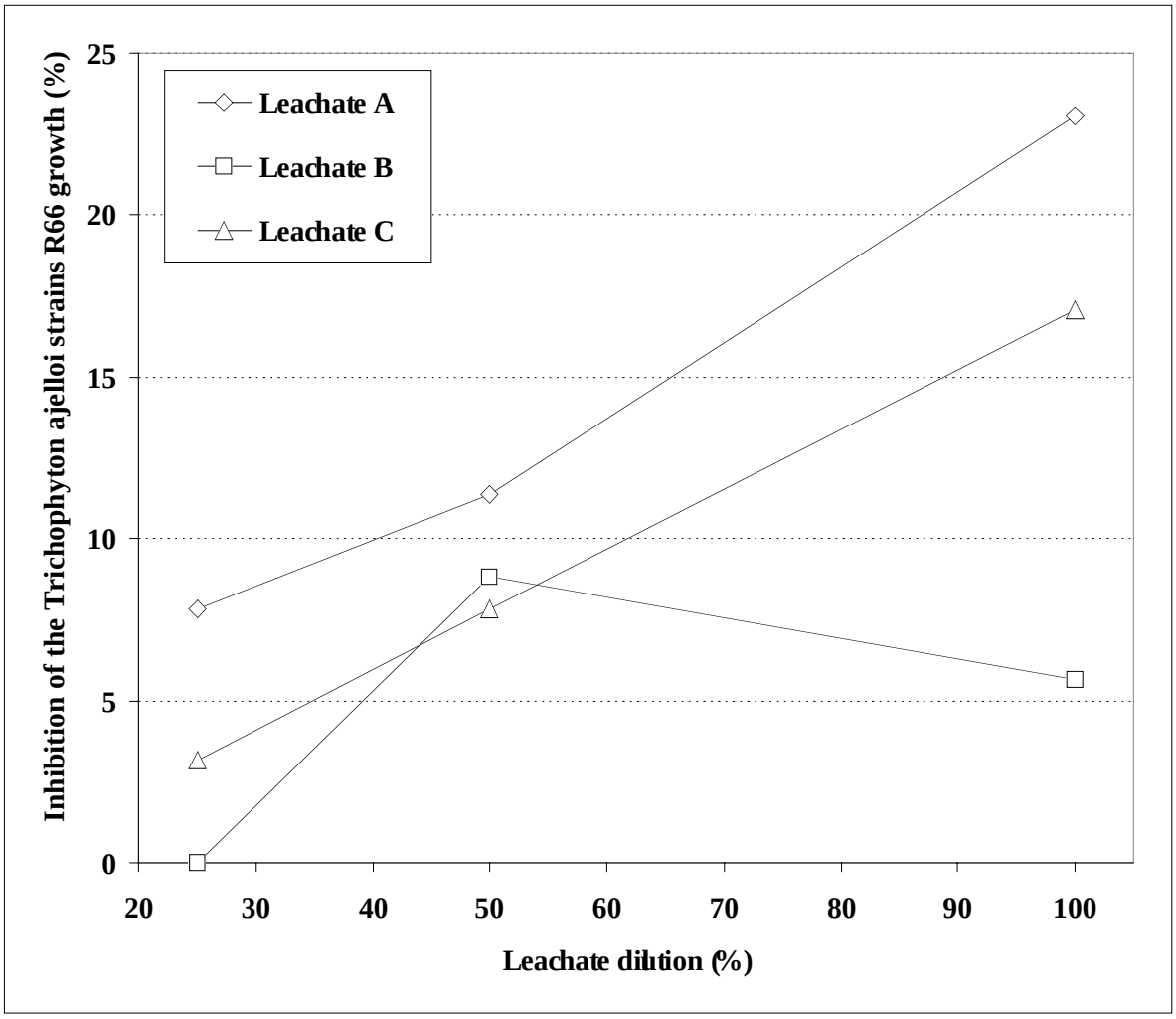

Fig. 22. Relationships between the inhibition of *Trichophyton ajelloi* strains R66 linear growth and leachate dilution

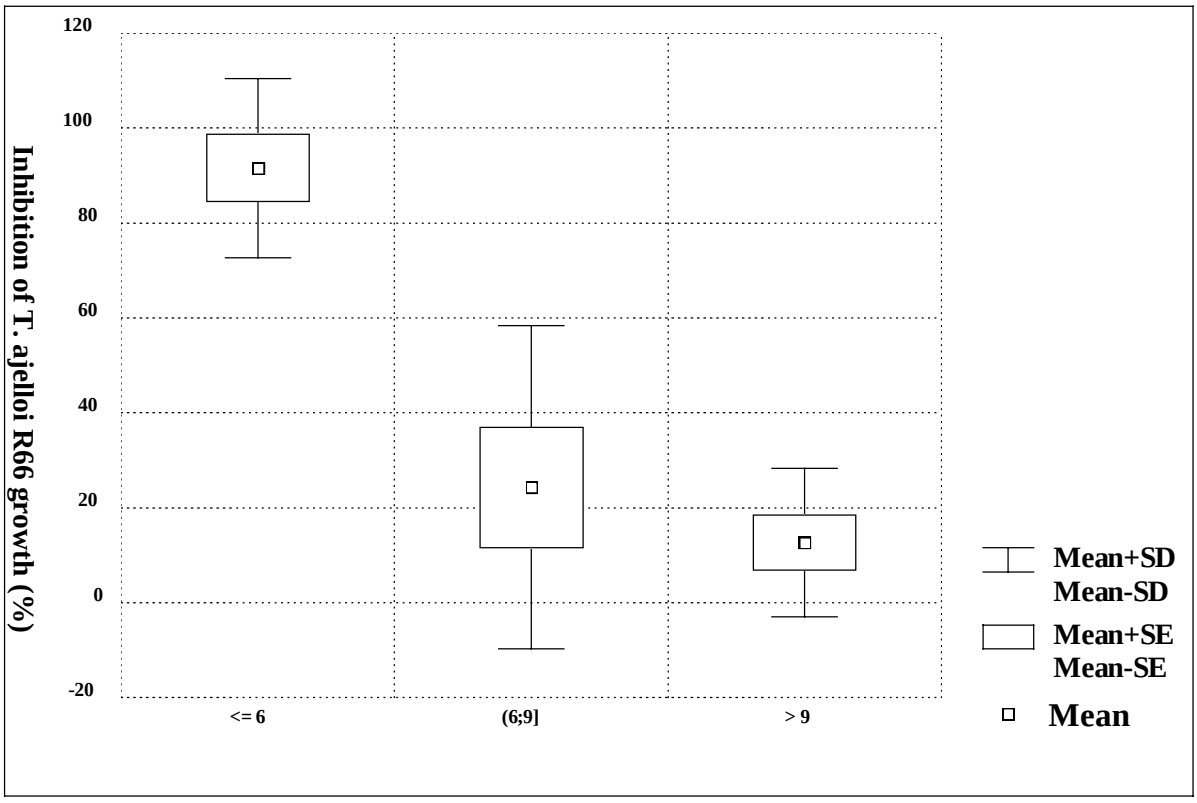

Fig. 23. Relationship between the inhibition of *Trichophyton ajelloi* strain R66 linear growth and the bioremediation time in months

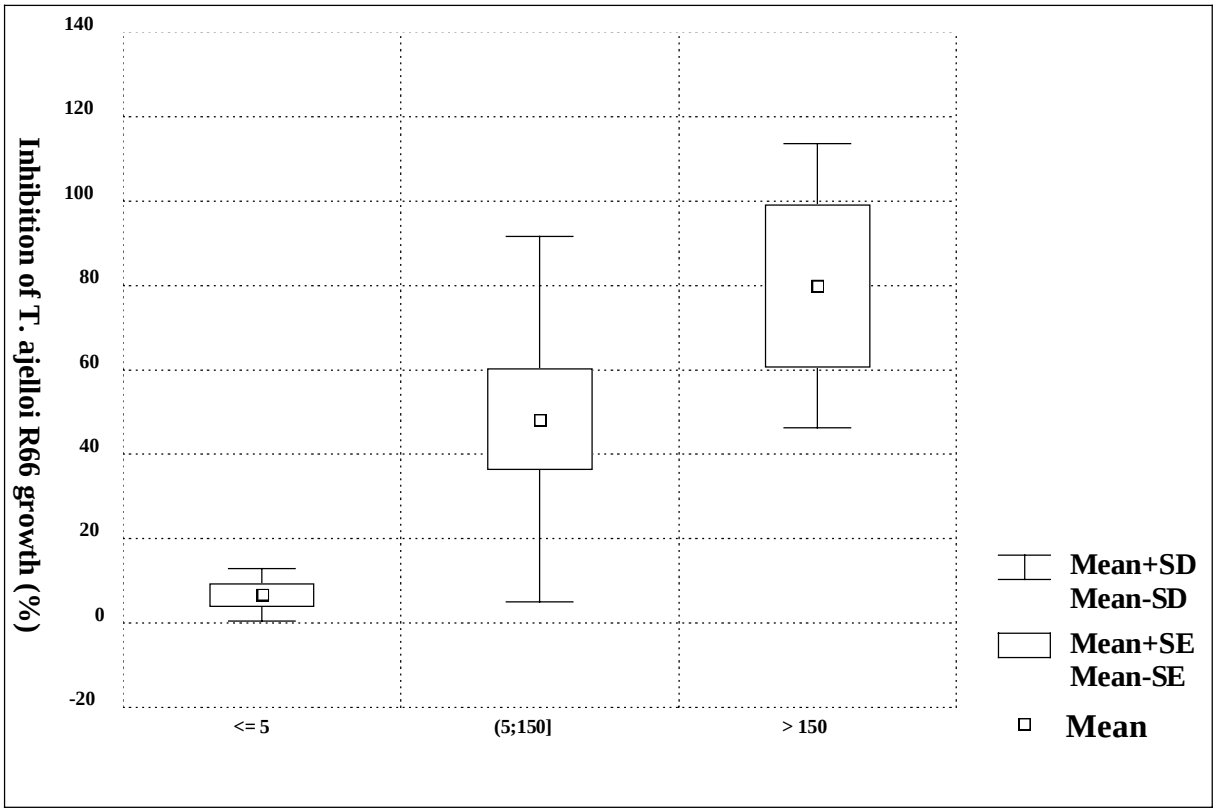

Fig. 24. Relationship between the inhibition of *Trichophyton ajelloi* strain R66 linear growth and TPOC concentrations in leachate

Table 1. Methods, standards & references for physico-chemical analyses

Parameter	**Methods**	**Standards & References**
pH in H_2O	Electrometric	PN ISO 10390 : 1997
pH in 1 M KCl	Electrometric	PN ISO 10390 : 1997
Conductivity CON	Cunductometric	PN ISO 11265 : 1997
Total Nitrogen N_{TOT}	Kjeldahl	ISO 11261 : 1995
Total Organic Carbon TOC	Tiurin	PN-91/Z-15005
Total Phosphorus P_{TOT}	Colorimetric	PN-91/C-04537.09
Available Phosphorus P_2O_5	Egner-Riehm	PN-R-04023 : 1996
Available Potassium K_2O	Egner-Riehm	PN-R-04022 : 1996
Total Sulfur S_{TOT}	Eschka	ISO 334 : 1992
$S\text{-}SO_4$	Bardsley & Lancaster	ISO 11048 : 1995
$N\text{-}NO_3$	Colorimetric	PN-87/C-04576.07
$N\text{-}NO_2$	Colorimetric	PN-73/C-04576.06
$N\text{-}NH_4$	Colorimetric	ISO 7150-1
PO_4	Colorimetric	PN-89/C-04537.03
Ca	Atomic Absorption Spectrometry (AAS)	PN-R-04024 : 1997
Mg	Atomic Absorption Spectrometry (AAS)	PN-R-04024 : 1997
TPH & TPOC	Infrared Spectrometry (FT-IR) - with extraction in CCl_4	PB-07:1999; PB-10:1999; PN-82/C-04565.01; EPA Method 3620, 1992; EPA Method 8440, 1995; PN-V-04007,1997
PAHs: Naphthalene Acenaphthene Fluorene Phenanthrene Anthracene Fluoranthene Pyrene Benzo(a)anthracene Chrysene Benzo(b)fluoranthene Benzo(k)fluoranthene Benzo(a)pyrene Benzo(g,h,i)perylene Dibenzo(a,h)anthracene Indeno(1,2,3-cd)pyrene	HPLC with fluorescence detection	PB-06:1999; PB-09:1999; ISO/FDIS 13877, 1998

Table 2. Physico-chemical data for determination of mineral NPK fertilization doses for the engineered biopile

Parameters	Units	Mixed soil samples from subsections:			
		I	II	III	IV
Moisture	%	18.19	18.42	17.40	17.62
Ignition losses at 600 ^{o}C	% d.w.	11.39	12.73	5.87	5.57
Total nitrogen N_{TOT}	% d.w.	0.128	0.118	0.097	0.133
Total organic carbon C_{ORG}	% d.w.	6.36	6.82	3.36	3.19
C:N ratio	-	49.69	57.80	34.64	23.98
Total phosphorus P_{TOT}	% P_2O_5 (d.w.)	0.401	0.256	0.225	0.214
Available phosphorus P_2O_5	mg P_2O_5/100 g d.w.	30.20	12.20	14.20	18.60
Available potassium K_2O	mg K_2O/100 g d.w.	18.90	17.30	19.20	24.80
pH in H_2O	-	6.49	6.61	7.05	6.90
pH in 1M KCl	-	7.13	7.05	7.23	7.24

d.w. – dry weight of soil

Table 3. Descriptive statististcs for selected physico-chemical parameters and dehydrogenase activity (TTC) for the bioremediation campaign (1997-2001) at the engineered biopile

Parameters	Units	N	Mean	Minimum	Maximum	Std. Dev.
pH in H_2O	-	552	6.95	2.64	9.94	0.88
Moisture	%	506	17.47	1.97	35.61	4.88
Dehydrogenase activity (TTC)	mg TPF/g d.w.	506	24.45	0	287.58	41.28
TPH	g/kg d.w.	506	16.02	0	115.98	19.37
TPOC	g/kg d.w.	506	23.32	0	168.64	26.87
POLAR	g/kg d.w.	506	7.3	0	55.43	9.19
PAH (Total)	mg/kg d.w.	414	3.93	0.05	47.53	5.11

Table 4. Descriptive statistics for microbiological parameters for the bioremediation campaign (1997-2001) at the engineered biopile

Parameters	Units	N	Mean	Minimum	Maximum	Std. Dev.
Number of bacteria (DAPI)	cells/g d.w.	460	3.07E+09	1.73E+06	2.48E+10	4.36E+09
Number of fungi (CW)	propagules/g d.w.	460	6.04E+07	1.35E+04	3.40E+09	3.38E+08
OIL_{23}	MPN/g d.w.	368	3.15E+11	5.91E+00	2.89E+13	2.25E+12
OIL_{37}	MPN/g d.w.	322	2.59E+11	6.38E+00	2.99E+13	2.37E+12
$NAPH_{23}$	MPN/g d.w.	368	2.10E+11	5.91E+00	2.88E+13	2.13E+12
$NAPH_{37}$	MPN/g d.w.	322	1.25E+11	5.91E+00	2.99E+13	1.69E+12

Table 5. Correlation coefficients (r) between selected physico-chemical parameters and dehydrogenase activity for the bioremediation period (1997-2001) at the engineered biopile

	pH in H_2O	Moisture	TTC	TPH	TPOC	POLAR	PAH
pH in H_2O	1.00*	0.05	0.05	-0.16*	-0.21*	-0.27*	-0.30*
Moisture		1.00*	0.27*	0.19*	0.18*	0.14*	0.17*
TTC			1.00*	0.10	0.09	0.06	0.07
TPH				1.00*	0.97*	0.74*	0.53*
TPOC					1.00*	0.87*	0.56*
POLAR						1.00*	0.53*
PAH							1.00*

* - correlations with $r \geq 0.14$ are statistically significant

Table 6. Correlation coefficients (r) between microbiological and selected physico-chemical parameters for the bioremediation period (1997-2001) at the engineered biopile

	pH in H_2O	Moisture	TTC	TPH	TPOC	POLAR	PAH
Bacteria (DAPI)	0.08	-0.06	-0.15*	-0.12*	-0.15*	-0.20*	-0.15*
Fungi (CW)	0.08	-0.05	-0.09	0.13*	0.14*	0.14*	0.08
OIL_{23}	0.06	0.00	-0.02	-0.01	-0.01	-0.03	0.07
OIL_{37}	-0.01	0.00	-0.04	-0.07	-0.08	-0.07	-0.06
$NAPH_{23}$	0.05	-0.03	-0.03	-0.05	-0.05	-0.05	0.21*
$NAPH_{37}$	0.00	0.04	0.00	-0.02	-0.02	-0.01	-0.03

* - correlations at $r \geq 0.12$ are statistically significant

Table 7. Changes in pH, moisture and dehydrogenase activity (means) during bioremediation at the engineered biopile

Month of bioremediation	Year	pH	Moisture	Dehydrogenase activity (TTC)
		-	(%)	(mg TPF/g d.w.)
0	1997	6.85	16.92	5.46
4	1998	6.58	20.92	31.69
7	1998	7.17	18.97	30.34
9	1998	7.19	16.83	5.34
13	1998	7.16	MD	MD
18	1999	6.41	16.23	23.55
21	1999	6.72	18.93	16.84
24	1999	6.64	9.36	19.83
39 (February)	2001	7.34	18.74	20.43
42 (May)	2001	7.28	16.17	8.08
44 (July)	2001	7.14	21.00	96.21
47 (October)	2001	6.95	18.16	11.16

MD – missing data

Table 8. Changes in the mean concentrations of petroleum-originating hydrocarbons in soil at the engineered biopile during the bioremediation period (1997-2001)

Month of bioremediation	Mean concentrations of petroleum hydrocarbons:			
	TPH	TPOC	POLAR	PAH (Total)
	g/kg d.w.	g/kg d.w.	g/kg d.w.	mg/kg d.w.
0	33.67	47.30	13.64	6.97
4	22.58	36.59	14.01	4.16
7	14.23	23.98	9.75	5.60
9	14.32	23.53	9.21	5.89
18	16.78	24.32	7.54	2.14
21	18.34	27.53	9.19	2.56
24	7.40	15.30	7.90	2.18
39 (February 2001)	14.88	17.03	2.15	3.44
42 (May 2001)	11.17	13.09	1.93	MD
44 (July 2001)	7.93	9.65	1.72	MD
47 (October 2001)	14.93	18.25	3.32	2.41

MD – missing data

Table 9. Petroleum hydrocarbon contaminant mass losses (%) and removal rates (g or mg/kg d.w./month) during bioremediation (1997-2001) at the engineered biopile

Data	Units	Hydrocarbon mass losses & removal rates for:		
		TPH	TPOC	PAH
Whole biopile	%	55.65	61.41	65.43
Passive section	%	75.55	77.71	67.90
Active section	%	44.02	51.79	63.93
Shallow layer	%	47.59	53.67	62.94
Deep layer	%	59.64	65.25	66.72
Whole biopile	g or mg/kg d.w./month	0.40	0.62	0.10
Passive section	g or mg/kg d.w./month	0.38	0.56	0.07
Active section	g or mg/kg d.w./month	0.42	0.69	0.12
Shallow layer	g or mg/kg d.w./month	0.23	0.36	0.06
Deep layer	g or mg/kg d.w./month	0.57	0.88	0.13

Table 10. The occurrence of keratinolytic and keratinophilic fungi in soil at the engineered biopile during bioremediation (1997-2001). Data obtained with the traditional Vanbreuseghem's method at 23 °C.

Fungal species and indices	Number of strains or species	Frequency (%)
Trichophyton ajelloi (Vanbreuseghem) Ajello	271	78.3
Teleomorph *Arthroderma uncinatum* Dawson et Gentles	6	1.7
Chrysosporium keratinophilum D.Frey ex Carmichael	19	5.5
Chrysosporium anamorph of *Aphanoascus reticulisporus*/*fulvescens*	8	2.3
Teleomorph *Aphanoascus reticulisporus* (Routien) Hubálek	6	1.7
Chrysosporium an. *Arthroderma curreyi* Berkeley	8	2.3
Myceliophthora vellerea (Sacc. et Speg) van Oorschot	6	1.7
Chrysosporium europae Sigler, Guarro & Punsola	6	1.7
Malbranchea fulva Sigler et Carmichael	5	1.4
Amauroascus mutatus (Quelet) Rammeloo	3	0.9
Microsporum sp.	2	0.6
Chrysosporium pannicola (Corda) van Oorschot et Stalpers	2	0.6
Scopulariopsis brevicaulis (Sacc.) Bain.	2	0.6
Microsporum gypseum (Bodin) Guiart et Grigorakis	1	0.3
Trichophyton terrestre Durie et Frey	1	0.3
Number of strains (NST)	346	-
Number of species (NSP)	13	-

Table 11. Differences in the means for fungal indices between active/passive sections and shallow/deep layers of the engineered biopile

Data	Mycological parameters		
	TAJ	NST	NSP
Passive section	0.83	1.15	11
Active section	0.63	0.72	7
Shallow layer	0.76	0.94	11
Deep layer	0.72	0.94	12

TAJ – frequency of *Trichophyton ajelloi*
NST – number of strains
NSP – number of species

Table 12. Changes in fungal indices (means) during bioremediation at the engineered biopile

Month of bioremediation	Year	Fungal indices at 23 ºC		
		TAJ	NST	NSP
4	1998	0.65	0.89	6
7	1998	0.83	1.15	7
9	1998	0.63	0.87	7
13	1998	0.63	0.67	3
18	1999	0.78	0.89	5
21	1999	0.74	0.83	4
24	1999	0.89	1.00	5
39	2001	0.74	1.22	7

TAJ – frequency of *Trichophyton ajelloi*
NST – number of strains
NSP – number of species

Table 13. The occurrence of soil keratinolytic and keratinophilic fungi in the engineered biopile. Results of the first 2001 sampling campaign. Incubation at four temperatures (23, 29, 33, and 37 C)

Fungal species	Temperature (°C)	Number of strains (NST)
Trichophyton ajelloi (Vanbreuseghem) Ajello	23	34
Teleomorph *Arthroderma uncinatum* Dawson et Gentles		3
Chrysosporium keratinophilum D.Frey ex Carmichael		9
Chrysosporium europae Sigler, Guarro & Punsola		3
Chrysosporium anamorf *Aphanoascus fulvescens/reticulisporus*		2
Teleomorf *Aphanoascus reticulisporus* (Routien) Hubálek		1
Malbranchea fulva Sigler et Carmichael		2
Myceliophthora vellerea (Sacc. et Speg) van Oorschot		1
Trichophyton terrestre Durie et Frey		1
NST_{23}		56
NSP_{23}		7
Trichophyton ajelloi (Vanbreuseghem) Ajello	29	26
Chrysosporium keratinophilum D.Frey ex Carmichael		21
Chrysosporium anamorf *Aphanoascus fulvescens/reticulisporus*		4
Teleomorf *Aphanoascus reticulisporus* (Routien) Hubálek		1
Microsporum gypseum (Bodin) Guiart et Grigorakis		4
Chrysosporium queenslandicum Apinis et Rees		3
Myceliophthora vellerea (Sacc. et Speg) van Oorschot		2
Chrysosporium anamorf *Aphanoascus clathratus* Cano et Guarro		1
NST_{29}		62
NSP_{29}		7
Chrysosporium keratinophilum D.Frey ex Carmichael	33	19
Pseudallescheria boydii (Shear) McGinnis et al.		13
Chrysosporium anamorf *Aphanoascus clathratus* Cano et Guarro		10
Chrysosporium queenslandicum Apinis et Rees		10
Chrysosporium anamorf *Aphanoascus fulvescens/reticulisporus*		5
Teleomorf *Aphanoascus reticulisporus* (Routien) Hubálek		2
NST_{33}		59
NSP_{33}		5
Pseudallescheria boydii (Shear) McGinnis et al..	37	8
Chrysosporium anamorf *Aphanoascus clathratus* Cano et Guarro		3
Chrysosporium anamorf *Aphanoascus fulvescens/reticulisporus*		1
Chrysosporium keratinophilum D.Frey ex Carmichael		1
Chrysosporium queenslandicum Apinis et Rees		1
NST_{37}		14
NSP_{37}		5
Total number of strains (Total NST)	-	191
Total number of species (Total NSP)	-	11

Table 14. Number of fungal strains and species isolated from the engineered biopile during the first 2001 sampling campaign at four temperatures (23, 29, 33 and 37 ºC)

Sections & layers	Total number of strains	Total number of species
Former passive section	135	11
Former active section	56	8
Shallow layer	95	11
Deep layer	96	9

Table 15. Physico-chemical characteristics of mixed soil samples from the engineered biopile examined with Microtox® Test System

Parameters	Units	Mixed soil samples no.							
		1	2	3	4	5	6	7	8
pH w H_2O	-	6.88	6.93	7.01	7.18	6.20	6.13	6.26	6.38
pH w 1M KCl	-	7.24	7.45	7.70	7.75	6.50	6.85	6.90	7.06
Conductivity	mS/cm at 25 ^oC	0.94	0.58	0.81	0.58	0.90	0.44	0.60	1.16
Moisture	%	3.37	4.87	3.83	3.62	3.94	5.17	4.54	5.43
$N\text{-}NO_3$	mg/kg d.w.	2.77	1.07	2.67	2.25	<0.03	<0.03	<0.03	<0.03
$N\text{-}NO_2$	mg/kg d.w.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
$N\text{-}NH_4$	mg/kg d.w.	87.17	65.41	59.80	56.13	50.70	55.46	52.85	52.01
Total Organic Carbon TOC	% d.w.	1.98	3.52	0.29	1.92	2.16	0.44	1.41	3.45
Total Nitrogen N_{TOT}	% d.w.	0.99	0.11	1.22	0.11	1.27	1.64	1.62	0.27
C:N	-	2.00	32.00	0.24	17.45	1.70	0.27	0.87	12.78
Total Phosphorus P_{TOT}	% d.w.	0.06	0.06	0.06	0.06	0.06	0.07	0.08	0.08
PO_4	mg/kg d.w.	23.00	29.00	26.00	24.50	10.00	9.00	12.50	10.00
Available Phosphorus P_2O_5	mg P_2O_5/100 g d.w.	12.16	17.68	18.79	15.52	14.41	19.81	13.85	19.34
Available Potassium K_2O	mg K_2O/100 g d.w.	21.90	13.70	14.20	11.20	14.00	13.10	13.80	11.90
Total Sulfur S_{TOT}	% d.w.	0.07	0.06	0.04	0.04	0.20	0.08	0.08	0.05
$S\text{-}SO_4$	% d.w.	0.03	0.04	0.02	0.04	0.08	0.02	0.02	0.03
Ca	mg/kg d.w.	4200	4215	8980	4055	2485	1875	3140	2985
Mg	mg/kg d.w.	793	371	1018	550	524	262	498	873
TPH	g/kg d.w.	7.88	4.27	0.74	0.88	13.77	7.75	9.68	3.42
TPOC	g/kg d.w.	9.08	5.59	1.07	1.06	16.96	10.28	10.83	4.18
Flouranthene	mg/kg d.w.	0.39	0.45	0.37	0.54	2.69	0.39	0.67	0.22
Pyrene	as above	0.48	0.39	0.33	0.43	2.52	0.50	0.60	0.19
Benzo(a)anthracene	as above	0.20	0.21	0.19	0.25	0.52	0.23	0.18	0.12
Chrysene	as above	0.09	0.08	0.11	0.16	0.16	0.14	0.07	0.04
Benzo(b)fluoranthene	as above	0.20	0.21	0.19	0.25	0.52	0.23	0.18	0.12
Benzo(k)fluoranthene	as above	0.09	0.08	0.11	0.16	0.16	0.14	0.07	0.04
Benzo(a)pyrene	as above	0.20	0.18	0.23	0.31	0.46	0.28	0.19	0.10
Dibenzo(a,h)anthracene	as above	0.08	0.07	0.05	0.06	0.19	0.09	0.20	0.07
Benzo(g,h,i)perylene	as above	0.19	0.20	0.19	0.21	0.37	0.25	0.27	0.13
Indeno(1,2,3-cd)pyrene	as above	0.21	0.27	0.24	0.32	0.34	0.44	0.23	0.19
PAHs Total	as above	2.12	2.15	2.01	2.69	7.92	2.70	2.66	1.22

Table 16. Concentrations of heavy metals in mixed soil samples 1-8 from the engineered biopile examined with the Microtox® Test System

Metals	Units	Concentrations of heavy metals in samples:							
		1	2	3	4	5	6	7	8
Cd	mg/kg d.w.	<2.09	<2.09	<2.09	<2.09	<2.09	<2.09	<2.09	<2.09
Pb	as above	121.6	57.0	39.8	46.0	70.8	50.9	61.7	48.7
Zn	as above	111.4	128.3	111.5	130.8	137.3	86.1	99.5	98.0
Cu	as above	21.8	25.2	25.3	29.1	26.7	23.6	44.1	23.8
Cr	as above	60.0	26.0	29.0	30.9	33.9	30.0	36.5	28.9
Ni	as above	169.6	22.4	24.3	24.6	28.6	28.7	41.5	25.7
Co	as above	6.9	6.6	8.4	8.1	8.4	8.1	11.5	7.9
Mn	as above	276.1	222.5	339.9	299.2	317.2	227.4	289.8	226.1
Fe	as above	17041	14878	16948	17872	19969	18206	27492	16389
Hg	as above	1.8	1.0	1.6	0.9	1.6	1.0	1.3	1.5
As	as above	6.3	6.0	6.4	7.0	9.2	7.1	10.6	8.5

Table 17. Physico-chemical characteristics of a leachate sample from the engineered biopile examined for ecotoxicity with the Microtox® Test System

Leachate	Unit	Value
pH	-	6.52
Conductivity	_S/cm w 25°C	676.7
$N\text{-}NH_4$	mg/L	0.12
$N\text{-}NO_2$	mg/L	<0.01
$N\text{-}NO_3$	mg/L	<0.05
PO_4	mg/L	<0.05
TPH	mg/L	1.05
TPOC	mg/L	2.97
Fluoranthene	_g/L	0.78
Pyrene	_g/L	0.49
Benzo(b)fluoranthene	_g/L	0.27
Benzo(k)fluoranthene	_g/L	0.16
Benzo(a)pyrene	_g/L	0.11
Dibenzo(a,h)anthracene	_g/L	0.02
Benzo(g,h,i)perylene	_g/L	0.09
Indeno(1,2,3-cd)pyrene	_g/L	0.19
PAHs Total	_g/L	2.11

Table 18. Results of the toxicity determination in mixed soil and leachate samples with the Microtox® Test System

Samples	Water extracts		Alcohol extracts	
	pH	EC_{50}	pH	EC_{50}
Leachate	6.62	> 100%	-	
Soil 1	6.60	> 100%	6.69	> 100%
Soil 2	6.65	> 100%	6.55	> 100%
Soil 3	6.76	> 100%	7.28	> 100%
Soil 4	7.23	> 100%	7.44	> 100%
Soil 5	5.93	> 100%	5.45	$EC_{50/5\ min}$ = 22.23 % $EC_{50/15\ min}$ = 19.30 % $EC_{50/30\ min}$ = 10.59 %
Soil 6	5.55	> 100%	5.28	$EC_{50/5\ min}$ = 30.01 % $EC_{50/15\ min}$ = 24.87 % $EC_{50/30\ min}$ = 21.71 %
Soil 7	6.52	> 100%	6.54	> 100%
Soil 8	6.24	> 100%	6.04	> 100%

Table 19. Monthly mean values of ambient air temperature (T), relative humidity (F), precipitation (P), days with precipitation (L) as well as values of WW index and ∑WW index in the period of I – IX.2001 in Bielsko-Biala

	I	II	III	IV	V	VI	VII	VIII	IX
T (^{0}C)	-0.8	0.4	4.1	7.5	14.5	14.3	18.7	18.9	11.7
F (%)	84	78	80	76	66	78	80	74	84
P (mm)	71.1	41.5	77.2	137.9	75.7	172.1	250.4	98.3	162.5
∑ WW (mm)	-	-	-	97.4	-44.0	116.8	172.4	14.3	121.8
WW_m	-	-	-	3.2	-1.4	3.9	5.6	0.5	4.1
WW_{max}	-	-	-	8.8 (22.04)	1.5 (31.05)	11.0 (23.06)	18.6 (26.07)	4.6 (10.08)	8.8 (16.09)
WW_{min}	-	-	-	-1.7 (4.04)	-4.4 (17.05)	0.3 (7.06)	-3.1 (15.07)	-3.8 (20.08)	0.7 (13.09)
Δ WW	-	-	-	10.5	5.4	10.7	21.7	8.4	8.1

I-IX – January-September 2001

Table 20. Monthly mean and amplitude of average daily temperature (in parenthesis) as a function of soil depth and temperature differences between the depth of 5 and 20 cm [ΔT (5-20)] and of 5 and 50 cm [ΔT (5-50)] in Bielsko-Biala

D (cm)	I	II	III	IV	V	VI	VII	VIII	IX
5	0.5 (4.5)	1.8 (6.4)	3.8 (7.7)	7.8 (12.9)	15.6 (7.6)	16.0 (10.4)	19.6 (9.3)	20.9 (10.5)	12.7 (5.9)
10	0.9 (3.7)	2.0 (5.9)	3.9 (7.2)	7.9 (11.7)	15.9 (8.1)	16.1 (10.0)	19.8 (9.1)	21.3 (10.2)	13.2 (5.4)
20	1.1 (3.1)	2.0 (5.1)	3.6 (6.6)	7.4 (9.9)	14.9 (6.2)	15.1 (6.0)	18.6 (7.2)	20.7 (7.4)	13.4 (5.4)
50	2.2 (2.7)	2.8 (4.1)	3.8 (4.1)	6.8 (6.3)	13.4 (4.5)	14.6 (5.1)	18.5 (4.7)	19.5 (4.0)	14.1 (4.2)
ΔT (5-20)	-0.6	-0.2	0.2	0.4	0.7	0.9	1.0	0.2	-0.5
ΔT (5-50)	-1.7	-1.0	0.0	1.0	2.2	1.4	1.1	1.4	-1.4

I-IX – January-September 2001

Table 21. Monthly mean and amplitude of noon (12^{00} GMT) temperature (in parenthesis) as a function of soil depth and temperature differences between the depth of 5 and 20 cm [ΔT (5-20)] and of 5 and 50 [ΔT (5-50)] in Bielsko-Biala

D (cm)	I	II	III	IV	V	VI	VII	VIIII	IX
5	0.8 (5.6)	2.4 (8.0)	5.4 (10.9)	10.4 (16.1)	19.2 (12.2)	18.9 (15.9)	22.3 (12.5)	25.2 (15.9)	14.8 (8.7)
10	1.0 (3.9)	2.1 (6.2)	4.5 (8.5)	9.0 (13.3)	17.2 (10.3)	17.2 (12.1)	20.8 (9.4)	22.9 (13.4)	14.1 (6.4)
20	1.1 (3.2)	1.9 (4.8)	3.7 (6.5)	7.5 (10.3)	15.1 (8.1)	15.2 (6.2)	18.7 (6.9)	20.8 (8.7)	13.4 (5.3)
50	2.3 (2.7)	2.8 (3.2)	3.8 (4.2)	6.8 (6.7)	13.3 (4.7)	14.6 (5.3)	18.4 (4.7)	19.6 (4.0)	14.1 (5.1)
ΔT (5-20)	-0.3	0.5	1.7	2.9	4.1	3.7	3.6	4.4	1.4
ΔT (5-50)	-1.5	-0.4	1.6	3.6	5.9	4.3	3.9	5.6	0.7

I-IX – January-September 2001

LEAD PHYTOEXTRACTION FIELD OPTIMIZATION

Introduction

Phytoextraction is an efficient soil cleaning process that is dependent upon the choice of plant species, the maintenance of plant growth and health, the production of biomass and the application of soil amendments. Amendments, mainly chelating agents, facilitate the amount of metal that plants can uptake from contaminated soil (Salt et al., 1995; Salt et al., 1998; Blaylock and Huang 2000; Ensley 2000). Since chelating agents can account for 60-70 % of total costs, a more efficient application method was employed in FY01.

A computer-driven dispenser, designed and tested by IETU during FY00 and used during the FY01 Project, resulted in amendment savings of about 35%. Parallel investigations conducted by Central European Advanced Technologies (CEAT) highlighted the importance of utilizing plant stress measurements to optimize harvest time and thus maximize metal uptake.

The FY01 project focused on field demonstration of phytoextraction activities, including computerized amendment application technology in conjunction with CEAT s Chlorophyll Fluorometer measurements, which helps to monitor plant stress and metal uptake levels. This investigation was done by the Technical University of Budapest (TUB), therefore the data is not reported here.

The aim of the FY01 Project was to combine the achievements of FY1996-2000 with further developments in the phytoextraction process. The following activities were performed during the project:

_ Site characterization (update),

_ Laboratory and field experiment (harvesting two crops),

_ Sampling and chemical analysis; and

_ Data summarizing and reporting.

Experimental scheme

Investigations were focused on three main aspects:

1. Testing various methods of establishing high biomass production;

2. Testing various amendment application schemes; and

3. Investigating metal uptake in relation to time after amendments application.

Laboratory experiment

The objective of the laboratory test was to select the most promising species for field investigations and to work out a protocol for customizing the phytoextraction process to fit particular conditions.

Method
In the laboratory, pots were filled with soil from a CF2 site and sunflower and mustard seeds were planted. The same number of seeds was planted into clean garden soil to grow seedlings. After two weeks, the seedlings were transplanted into larger pots containing contaminated soil from the CF2 site and immediately treated with amendments.

Amendments were applied and plant samples were collected according to the scheme presented below.

Treatment	Sunflower		Mustard	
	planted	transplanted	planted	transplanted
1	• Control (no amendment)			
2	• 5 mmol EDTA + acetic acid + Roundup			

One week after amendment application, plants were harvested and analyzed. Five replications were performed for each treatment.

Results
No significant differences were observed in laboratory experiments comparing seeding and transplanting techniques for plant introduction into a contaminated site and during experiments comparing various species and metal uptake differences (see Figure 1). Experiment results showed no increase in phytoextraction from the use of seedlings rather than seeds.

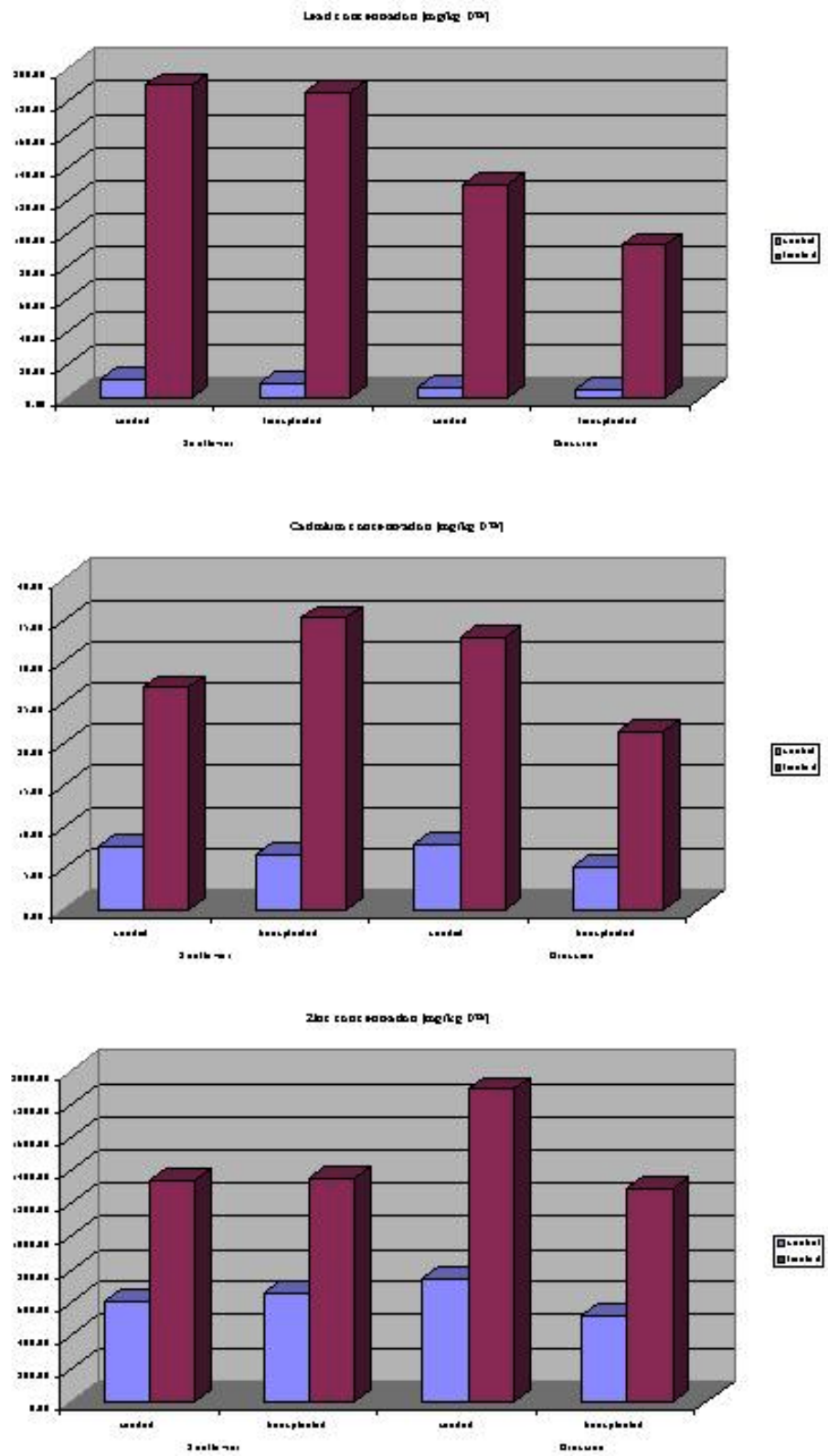

Figure 1. Metal concentration in plants depending on planting method

Based on data from the laboratory experiments, since transplanting not help, use of seeds was chosen for planting technique, due to less cost and simplicity, and white mustard (*Sinapis alba* cv. Nakielska) was selected for the field investigations.

Field investigations

Method

Plots establishing

The experiment was carried out on the CF2 experimental field used for the research during past years in the Project. The plots 2x5 meters in three replications were selected on the field when the plants achieved the stage of development suitable for amendment application. The general criteria for area selection was good, consistent plant development.

The plots were divided into two subplots after the last dose of amendment A (EDTA) application. One part was treated with amendment B (Roundup) and another left untreated.

Before amendment application the soil was sampled and analyzed for metal content for each plot.

After Crop 1 harvest the plots were cultivated and again planted with mustard (*Sinapis alba* cv. Nakielska) for Crop 2.

Seed bed preparation and planting

The soil was cultivated in the spring following regular agricultural practices and then cultivated with rototiller after Crop 1 harvesting. Before planting (Crop 1 and Crop 2), proper fertilizer doses were calculated on the basis of chemical analyses and with software (NAV2) used by the Polish agricultural services.

Amendment application and harvesting

It was demonstrated that the application of EDTA in three steps enhanced heavy metal accumulation in plant leaves to a greater extent than the one step treatment (DOE 2000). This is important since stems and leaves (the shoots of a plant) will be harvested in the phytoextraction process and analyzed for metal uptake. When the plants reached the appropriate developmental stage, amendments were applied according to the following table.

Treatment Code	Species - Mustard (*Sinapis alba* cv. Nakielska) Method of canopy establishing - Planted
Control	Control (no amendment)
A1	5 mmol EDTA + acetic acid
A2	5 mmol EDTA + acetic acid + Roundup
B1	2.5 mmol EDTA +acetic acid (first dose) 2.5 mmol EDTA + acid
B2	2.5 mmol EDTA +acetic acid (first dose) 2.5 mmol EDTA + acid +Roundup
C1	1.7 mmol EDTA + acetic acid (first dose) 1.7 mmol EDTA + acetic acid (second dose) 1.7 mmol EDTA + acetic acid
C2	1.7 mmol EDTA + acetic acid (first dose) 1.7 mmol EDTA + acetic acid (second dose) 1.7 mmol EDTA + acetic acid + Roundup

Entire plants were sampled at two-day intervals starting from the time of the first amendment application (Days 3, 5, 7 and 9 after amendment application).

Plant sampling

The sample was taken from each plot. Each time, 6-7 plants were selected randomly from the plot and harvested. The entire plant was taken for sampling, and then roots were separated. The stems and leaves were cut, then dried in 60 ^{0}C and grained through 2mm sieve.

The samples were analyzed with X-Met 920 XRF analyzer.

Results of the field experiment

Soil analysis — revision

The experimental plots were located in the middle of the CF2 field used for the FY-00 Project. The soil on each plot was analyzed for corrections and finalization of data. Lead and zinc concentrations are given in Table 1. There are no significant differences among samples in the blocks.

Table 1. Metal concentration in soil of experimental plots

Block	Plots	Pb	Zn
		[mg/kg]	
1	1	496.43	934.42
	2	541.04	1011.08
	3	572.47	1147.88
	4	528.72	990.73
	Aver.	**534.67**	**1021.03**
	SD	*27.24*	*78.44*
2	1	458.61	749.53
	2	451.74	850.31
	3	482.86	939.97
	4	473.60	844.49
	Aver.	**466.70**	**846.08**
	SD	*12.23*	*67.38*
3	1	580.63	1218.47
	2	552.97	1341.88
	3	534.56	1445.50
	4	557.80	1304.12
	Aver.	**556.49**	**1327.49**
	SD	*16.41*	*81.49*

Block 2 is less contaminated with lead and zinc than Block 1 and Block 3. The data shows that the soil is moderately contaminated and suitable for phytoextraction.

Plant material

Visual observations of the first and second crop were made after amendment application. Both crops were less damaged when the 5 mmol dose of EDTA was divided into three applications (see figures 2, 3 and 4). Plants appeared to be even less damaged when Roundup was not applied.

Figure 2. Comparison of plants with A1 and A2 treatments (A1 — 1 dose EDTA without Roundup; A2 — 1 dose EDTA with Roundup)

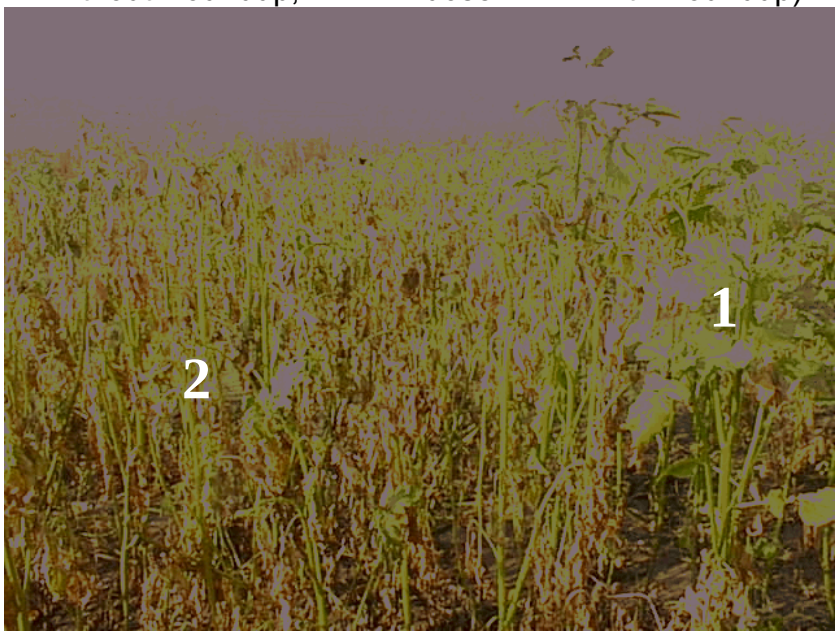

Figure 3. Comparison of plants B1 and B2 treatments (B1 — 2 doses EDTA without Roundup; B2 — 2 doses EDTA with Roundup)

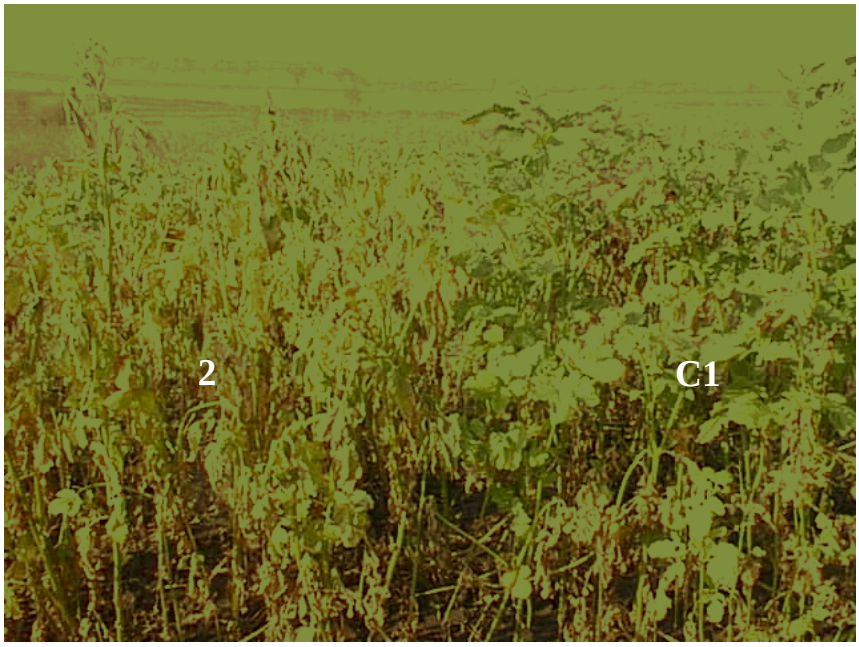

Figure 4. Comparison of plants C1 and C2 treatments (C1 — 3 doses EDTA without Roundup; C2 — 3 doses EDTA with Roundup)

Analytical data from the first crop showed that the maximum concentration of each amendment application regimen was observed on the fourth day after treatment. The second harvest would confirm the maximum metal uptake by plants during the four-day process.

Table 2 shows the average values for each day of sub-plot sampling. The starting data is represented by one sample taken from the plot.

Several independent factors influenced the samples, for example individual plant behavior, metal concentration in the soil, sub-plot, etc. These factors were taken into account when determining the standard deviation value calculated for each replication. Figure 5 shows median values for lead concentration in experimental plants.

Table 2. Average lead concentration in mustard plants in days after amendment application (Crop 1)

Sampling day	Lead concentration in plant dry weight													
	A1		A2		B1		B2		C1		C2		Control	
	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD
1	37.84	16.44			35.73	16.20			28.75	11.86			31.01	5.44
2	101.43	53.01	75.98	-	72.87	26.44	72.87	26.44	59.39	35.55	59.39	35.55	35.51	-
3	149.90	86.67	114.11	51.03	187.01	36.14	161.97	-	160.45	124.96	160.45	124.96	37.75	-
4	209.20	80.52	174.42	65.03	352.43	159.46	251.06	141.50	239.11	167.27	144.51	-	38.88	-
5	178.95	-	137.39	-	244.35	-	138.69	41.10	207.88	76.12	128.57	74.95	40.00	17.11
6	148.70	85.88	100.35	61.54	136.28	90.38	122.78	-	125.20	125.20	172.90	89.31	41.76	-
7	110.63	-	80.32	-	99.58	-	114.83	-	120.55	-	125.80	-	42.64	-
8	72.56	27.25	60.29	27.54	62.87	31.40	106.88	50.65	115.89	53.49	78.70	29.91	43.51	21.48

A1 — 1 dose EDTA without Roundup
A2 — 1 dose EDTA with Roundup
B1 — 2 doses EDTA without Roundup
B2 — 2 doses EDTA with Roundup
C1 — 3 doses EDTA without Roundup
C2 — 3 doses EDTA with Roundup
1 Red numbers in the table are estimated averages values
SD — standard deviation of n=3.

The highest lead concentration in plants was found when EDTA was applied in two doses; Roundup did not significantly affect the lead concentration (see figures 5 and 6).

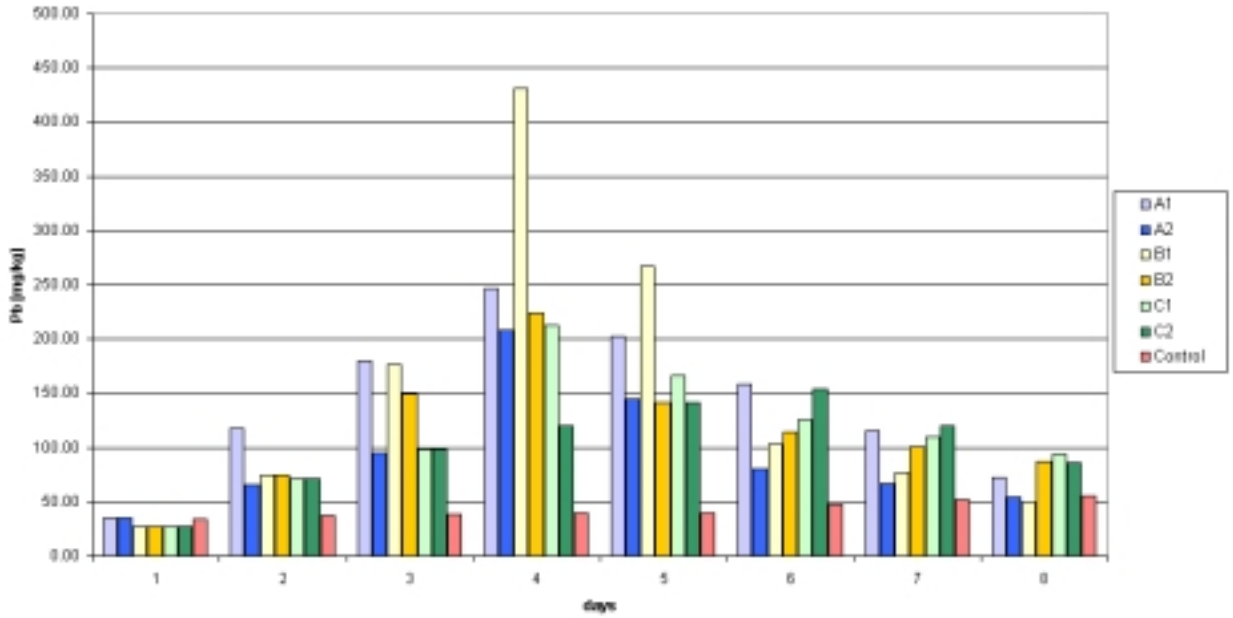

Figure 5. Median lead concentration in above ground plant parts following amendment application (Crop˚1).

The statistical analysis for influences of Roundup on lead content in plants showed that there was no significant difference (see Figure 6).

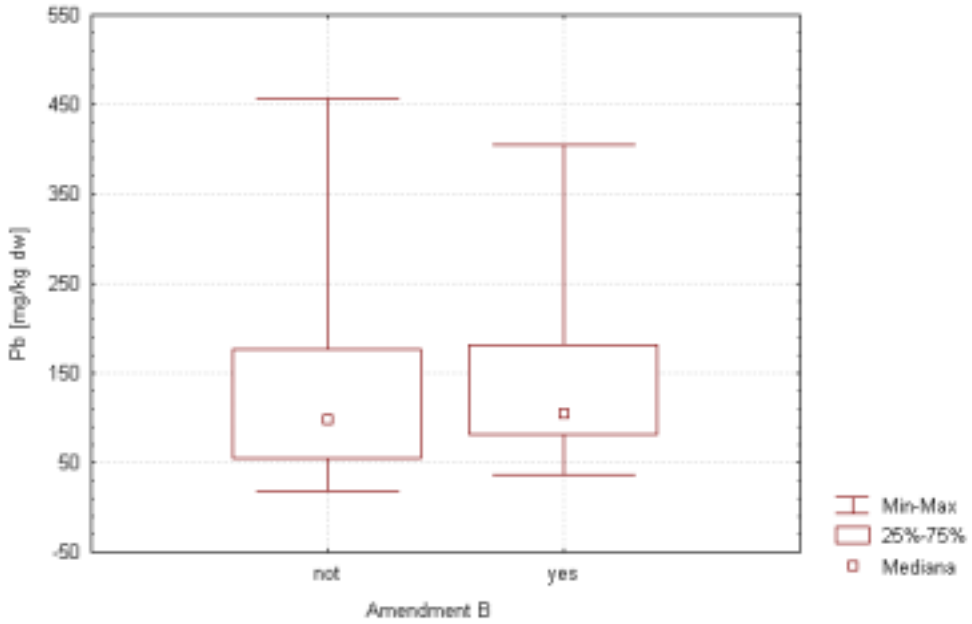

Figure 6. Influence of amendment B (Roundup) on lead uptake (Crop 1)

The statistical analysis completed for changes of lead content in relation to the time are presented on Figure 7.

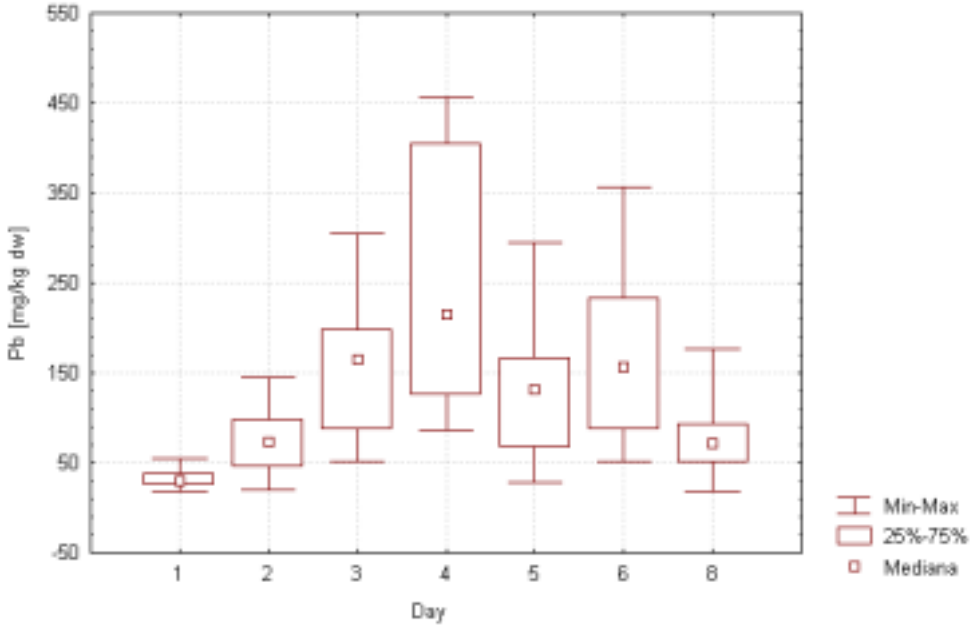

Figure 7. Changes of lead concentration in relation to time after amendment application (crop 1)

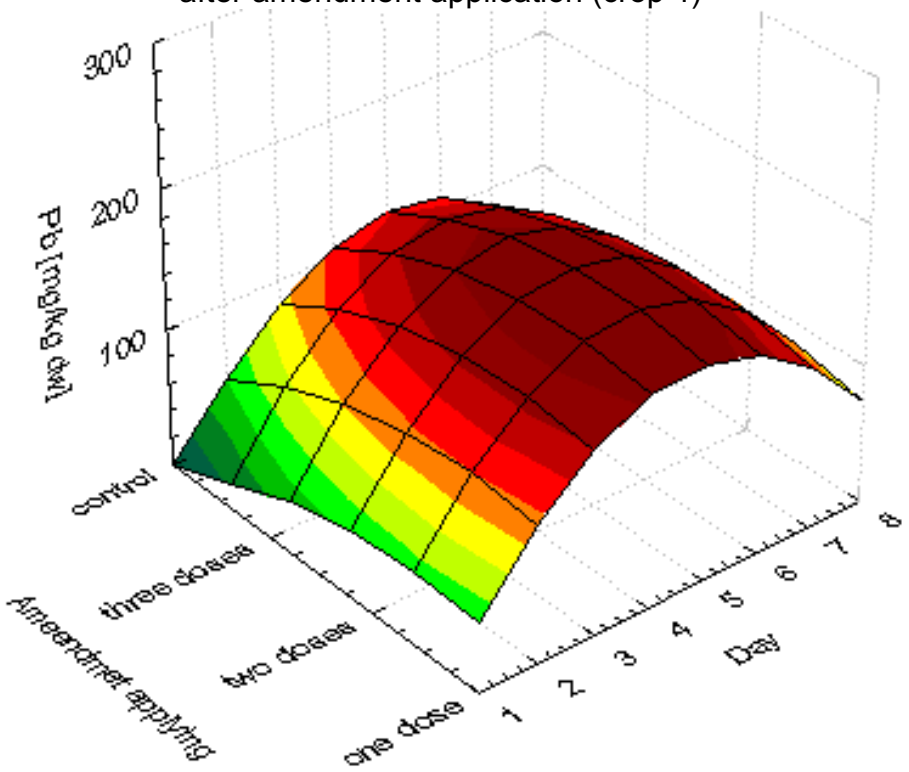

Figure 8. Datum of dispersion of lead concentration in time according to amendment application method

Analogous results were obtained for zinc concentration in plants (see Figure 8). A positive influence of Roundup was observed on zinc concentration in plants.

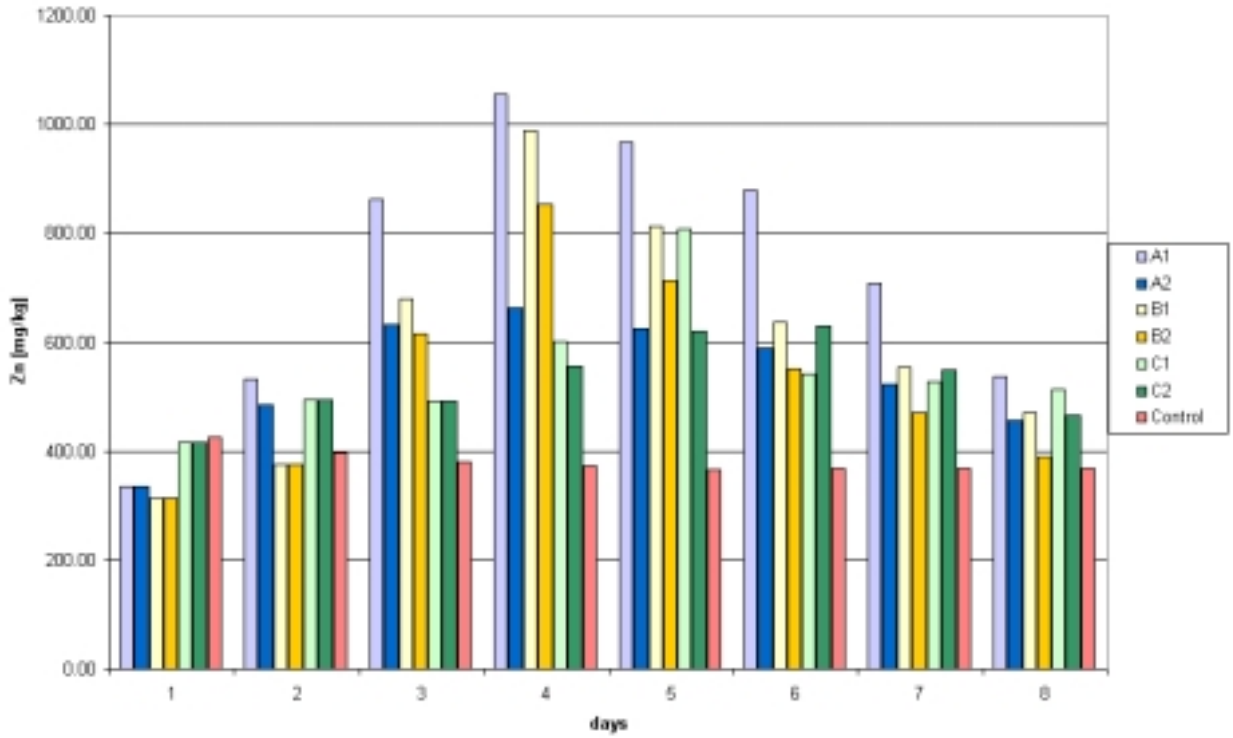

Figure 9. Median zinc concentration in plants
following amendment application (Crop 1)

At the end of September, the second experimental crop was treated and harvested according to the previously mentioned schedule and with the same experimental parameters used for the first crop.

Data indicated maximum metal concentration in plants for the standard regimen (A1) on the sixth day after amendment application. Amendment B (Roundup) did not significantly influence lead concentration (see Figure 10) when EDTA was applied in more than one dose. Lead uptake when EDTA was applied one time was decreased when amendment B was added. These results are similar to those obtained from crop˚1. With the first crop, the highest lead concentration in plants occurred only four days after amendment application; however, during the second crop, the highest lead concentration occurred 6-7 days after amendment application. This difference can be related to the dynamics of plants growth at different times during the vegetation period.

The important is that the maximum concentration of lead can be reached between 4-7 days after amendment application and in Spring the period between application of amendment and harvest ought to be shorter than regular and longer in Autumn. The role of Roundup is not clear.

Table 3. Average lead concentration in mustard plants following amendment application (Crop˚2)

Sampling day	Lead concentration in plant dry matter													
	A1		A2		B1		B2		C1		C2		Control	
	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD	mg/kg	SD
1	46.47	27.83			47.72	16.82			53.76	5.36			31.82	12.94
2	91.26	34.95	91.26	91.26	77.20	22.18	77.20	77.20	63.18	17.68	63.18	63.18	55.91	-
3	182.43	82.88	121.90	10.54	96.58	23.47	96.58	96.58	76.59	17.60	76.59	76.59	67.96	-
4	128.69	22.59	105.97	6.92	98.76	25.30	94.71	5.17	89.91	7.23	89.91	89.91	73.98	-
5	183.24	-	131.36	-	169.15	21.52	128.48	39.18	130.76	30.22	130.67	36.85	80.01	12.16
6	237.80	37.95	156.75	8.43	179.45	-	137.03	-	185.16	17.35	114.80	16.81	68.10	-
7	219.60	-	147.08	-	189.75	93.92	145.59	31.87	162.26	-	120.77	-	62.15	-
8	201.40	89.85	137.42	49.81	173.13	-	167.15	-	139.36	16.31	126.75	26.89	56.19	16.86

A1 — 1 dose EDTA without Roundup
A2 — 1 dose EDTA with Roundup
B1 — 2 dose EDTA without Roundup
B2 — 2 dose EDTA with Roundup
C1 — 3 dose EDTA without Roundup
C2 — 3 dose EDTA with Roundup
1 Red numbers in the table are estimated averages values
SD — standard deviation of n=3.

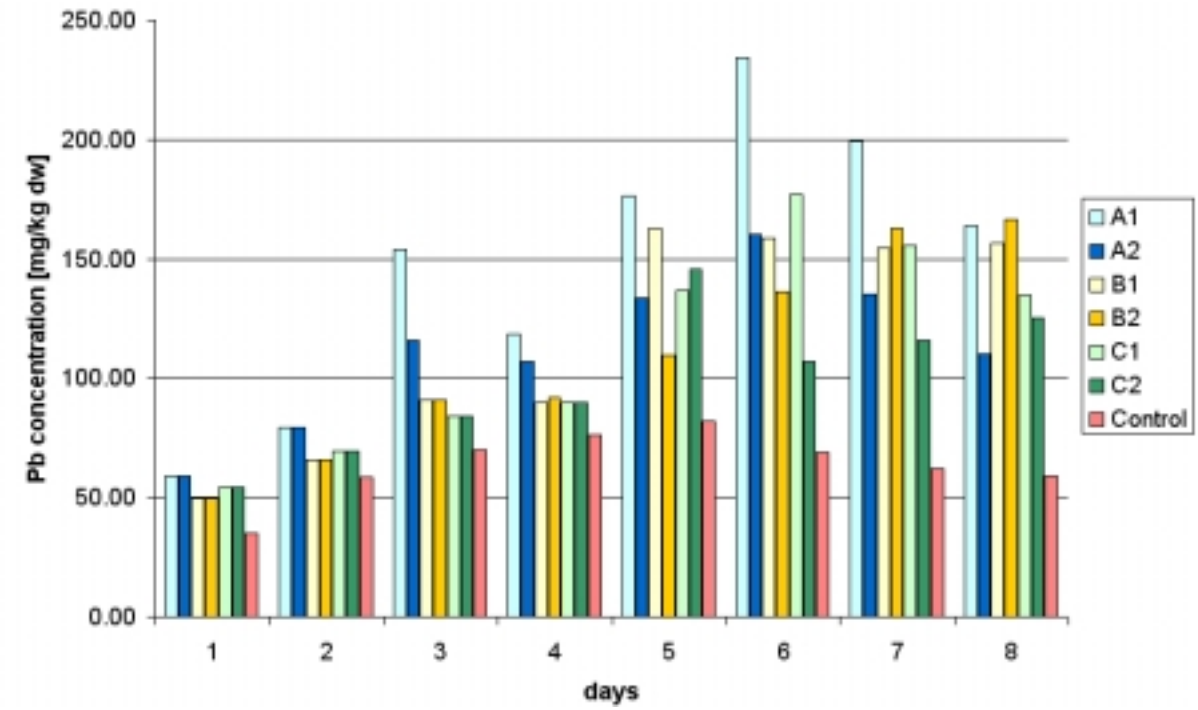

Figure 10. Median lead concentration in plants over time after amendment application (Crop˚2)

There is no effect of amendments on zinc uptake. There is a slight increase of zinc concentration on the 6th day (Figure 11).

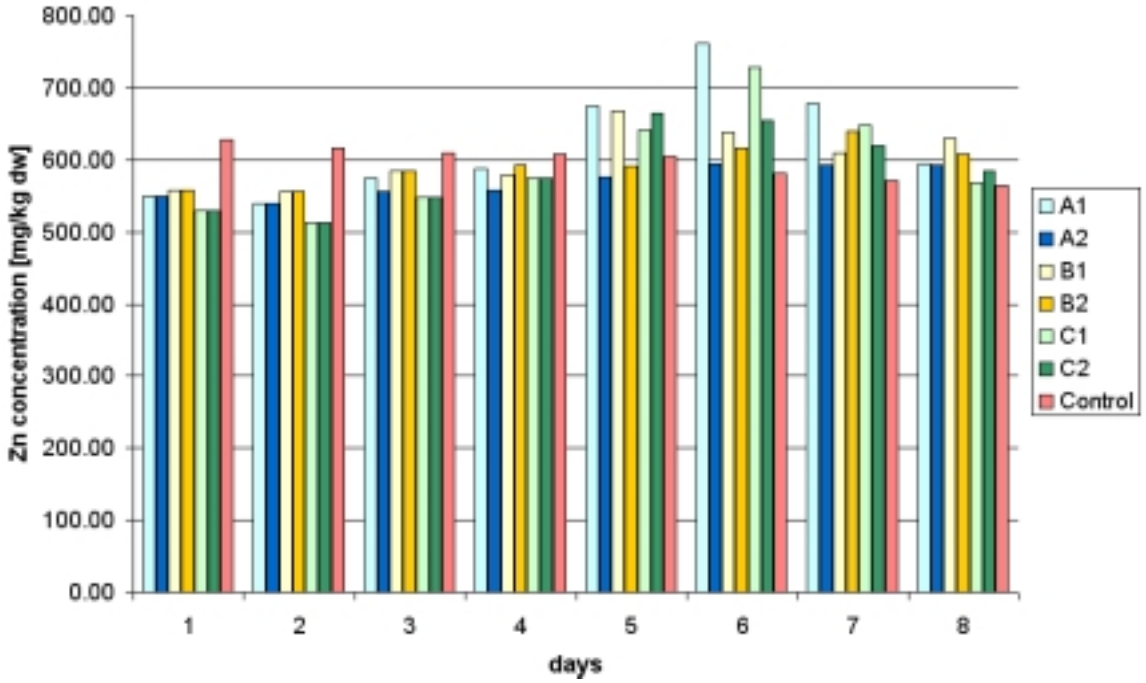

Figure 11. . Median zinc concentration in plants over time after amendment application (Crop˚2)

Conclusions

1. Laboratory studies showed no significant difference in metal (Pb and Zn) uptake between seeding and/or transplanting techniques for plant introduction into CF2 soil.

2. The two species used in experiments [mustard (*Sinapis alba* cv. Nakielska) and sunflower (*Helianthus annus)*] did not differ significantly in metal uptake ability.

3. Results indicate maximum lead concentration for each amendment application regimen on the fourth day for Crop 1 and sixth day for Crop 2 following amendment application.

4. Maximum lead concentration in plants was observed when amendment A (EDTA) was applied in two doses with Crop 1, and one dose with Crop 2.

5. Amendment B (Roundup) did not significantly improve lead uptake.

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PHYTOREMEDIATION TREATABILITY STUDIES FOR DOE DEPLOYMENT

Abstract

This study included experiments in which several plant species were evaluated for their ability to remove lead from firing range soils. The study also evaluated amendment application protocols, plant growth dynamics and lead uptake rates.

Three plant species were tested in the current study: sunflower (*Helianthus annuus* cv. Albena), *Ricinus communis* and white mustard (*Sinapis alba* cv. Nakielska). Plants were transplanted or seeded directly to firing range soil.

Soil amendments (EDTA and acetic acid) were applied as four treatments: Control (no amendment added); 5 mmol EDTA, 5 mmol acetic acid and amendment B (Roundup); 5 mmol EDTA (2.5 mmol EDTA in two doses), 5 mmol acetic acid (2.5 mmol acetic acid in two doses) and amendment B (Roundup); 5 mmol EDTA (1.7 mmol EDTA in three doses), 5 mmol acetic acid (one dose) and amendment B (Roundup).

Among the tested treatments, the most effective amendment application treatment for lead, cadmium and zinc accumulation in plant shoots was EDTA and acetic acid applied in one dose. Lead concentration was highest in shoots of white mustard seeded directly into the soil (16,500 mg/kg d.w.). Metal phytoextraction was higher in transplanted white mustard compared to white mustard seeded directly into contaminated soil, because of higher biomass production by transplanted plants. Seeded sunflower cv. Albena extracted more lead than seeded white mustard. High phytoextraction coefficients (between 16.7 and 24.7) were found in all species and treatments. These coefficients are higher than any previously reported and are probably due, in part, to the high degree of bioavailable lead in the target soils. These phytoextraction coefficients should give high phytoextraction of Pb from firing range soil at field scale.

Introduction

Soil at firing ranges contains bullets of various origin, construction and materials including lead, copper, zinc, antimony, ceramic and other various alloys (Landsberger et al., 1999). Firing range soil generally is contaminated to a depth of 20 cm (J rgensen and Willems, 1987; Landsberger et al., 1999).

Metallic lead reacts quickly with soil components and atmospheric media and forms various compounds, which may exert detrimental effects on soil and other environmental components (J rgensen and Willems, 1987). Metallic lead can become oxidized and transformed into a number of forms including bioavailable lead compounds that may be taken up by plants from soil solution (J rgensen and Willems, 1987; Rooney et al., 1999). Therefore, soil characterization is necessary to determine the horizontal and vertical distribution of this contaminant.

During FY01, the IETU conducted a Treatability Study that included laboratory plant uptake investigations on soil from a firing range site. The site was contaminated with lead and other metals as a result of historic firing range activity. The goal of the study was to determine the applicability of phytoremediation to the site, to select the most promising plant species given existing conditions and to identify the best amendment application protocol.

The studies were conducted under conditions of controlled light intensity, air humidity and temperature.

The studies were carried out in two steps:

1. Soil Characterization — chemical analysis to determine the concentration and speciation of elements; and

2. Treatability Studies - growth chamber experiments to evaluate the growth potential of selected plant species and to determine soil amendment conditions to optimize plant growth and heavy metal uptake.

The Treatability Study included an experiment in which the most promising plant species (based on earlier experiments and phytoremediation capability) were evaluated for applicability to the firing range site conditions. Additionally, optimal amendment application protocols were determined along with plant growth dynamics on the target soil and lead uptake from the soil by examined species.

Treatability Study

Soil samples for the Treatability Study were taken from an abandoned firing range located in Tarnowskie G ry (about 40 km from Katowice). The site belongs to the

Polish Army and was used as a general purpose firing range for many years. Further details are classified.

All soil analyses were conducted on air-dried soil. Initial samples were analyzed from potential sub-sites within the firing range - A and B. The copper concentration at Site B was 277.3 mg/kg soil, whereas at Site A, the copper concentration was 57.3 mg/kg soil. Since copper is known to be phytotoxic at concentrations above 100 mg/kg soil (Kabata-Pendias and Pendias 1999), Site A was chosen for further characterization. 100 kg of soil was collected from the site. Before transporting the soil from the site, large bullet fragments and stones were removed mechanically by sieving the soil with a 4 mm screen. About 4.9 kg of bullets per 100 kg of soil were recovered by this mechanical separation at Site A (Figure 1).

Figure 1. Bullets recovered from the soil at Site A

Physical and chemical properties of the soil are presented in tables 1-4.

Table 1. Total concentration of Pb, Cd, Zn and Cu (mg/kg) in soil samples from Site A after bullets removal (AAS, 10 % HNO_3 extraction). Values are means – SD (n=8).

Pb (mg/kg)	**Cd (mg/kg)**	**Zn (mg/kg)**	**Cu (mg/kg)**
669.0 ± 114.5	1.32 ± 0.43	55.2 ± 9.4	57.3 ± 4.8

Table 2. Total concentration of Mn, Fe, Cr, Mg and Ca (mg/kg) in soil samples from Site A after bullets removal (ICP analysis, 10 % HNO_3 extraction). Values are means – SD (n=5).

Mn (mg/kg)	**Fe (mg/kg)**	**Cr (mg/kg)**	**Mg (mg/kg)**	**Ca (mg/kg)**
21.20 ± 3.20	323.5 ± 46.2	0.241 ± 0.065	57.2 ± 4.1	587.2 ± 60.3

Table 3. Electrical conductivity and pH of soil samples from Site A. Values are means – SD (n=3).

EC (1:1) μ s/cm^2	**pH (KCl)**	**PH (H_2O)**
58.2 ± 1.4	5.16 ± 0.09	5.87 ± 0.12

Table 4. Soil texture of soil samples from Site A

Sample No.	Percent of total mass							
	Silt				clay <0.002	Sand		
	Fraction 0.10-0.05	Fraction 0.05-0.02	Fraction 0.020-0.005	Fraction 0.005-0.002		Coarse	Medium	Fine
1	7	2	3	2	4	29.42	24.00	27.58
2	7	5	5	3	3	25.36	24.99	27.65
3	6	5	2	4	2	23.58	21.42	30.00

Based on soil texture analysis (Table 4), the soil was classified as a light, loamy sand with the following macronutrient contents:

ÿ Total N = 0.043 (%);

ÿ Available phosphorus = 0.49 mg P_2O_5/100 g; and

ÿ Available potassium = 5.7 mg K_2O/100 g K (mg/kg).

The type and quantity of fertilizer applied to the experimental pots was based on soil fertility analysis and standard agricultural practices.

Based on previous experiments (data not published), sunflower (*Helianthus annuus* cv. Albena), white mustard (*Sinapis alba* cv. Nakielska) and *Ricinus communis* were chosen for the treatability study. The transplanting procedure described by Wu et al.

(1999) and Cooper et al. (1999) was used, since seedling growth could be affected adversely by high soil metal concentrations. *Ricinus communis* and white mustard were transplanted into the pots with contaminated soil after growth in clean soil (Wu et al., 1999). White mustard was transplanted into the pots after 3 weeks growth in clean soil, whereas *Ricinus communis* was transplanted after 8 weeks. This difference between the age of white mustard and *Ricinus communis* seedlings was connected with slow *Ricinus communis* growth. Seedlings in the same stage of growth were transplanted. Sunflower (*Helianthus annuus* cv. Albena) and white mustard (*Sinapis alba* cv. Nakielska) were seeded directly into pots with contaminated soil.

The following plants were used in the study:

ÿ *Ricinus communis* - one plant was transplanted into each pot with 800 g of contaminated soil,

ÿ white mustard - three plants were transplanted into each pot with 330 g of contaminated soil,

ÿ white mustard - seven plants were grown (seeded directly to the soil) in each pot with 330 g of contaminated soil,

ÿ sunflower - four plants were grown (seeded directly to the soil) in each pot with 330 g of contaminated soil.

The difference between the number of plants used in pot experiments was the result of differences in biomass of seedlings. 330 g of contaminated soil was used for white mustard and sunflower pot experiment, because this quantity of soil was sufficient to support growth of this plant species. 8 week old *Ricinus communis* produced higher biomass and required 800 g of contaminated soil for transplanting.

Amendment application

It was demonstrated in experimental investigations carried out with a Chlorophyll Fluorometer (made by CEAT, Budapest, Hungary) that the application of EDTA in three steps can enhance heavy metal accumulation in plant leaves to a greater extent than the one step treatment (DOE, 2000). Based on these results, the following treatments were used:

ÿ Control - (no amendment added);

ÿ 5 mmol EDTA + 5 mmol acetic acid + Roundup;

ÿ 5 mmol EDTA (2.5 mmol EDTA in each of two doses) + 5 mmol acetic acid (2.5 mmol acetic acid in each of two doses) + Roundup; and

ÿ 5 mmol EDTA (1.7 mmol EDTA in each of three doses) + 5 mmol acetic acid (one dose, applied in a single dose in conjunction with the first dose of EDTA) + Roundup.

Each treatment was tested in five replications.

Chemical Analysis

Soil analysis

To determine total lead, cadmium and zinc concentrations, the soil samples were ground with a porcelain mortar, sieved trough a 0.25 mm screen, and extracted by shaking for 1 h in 10% HNO_3. The resulting solution was filtered with filter paper (medium velocity of filtration, PPH POCh , S.A., Gliwice, Poland). The metal content was determined with flame AAS or ICP spectrometer (Varian SpectrAA 300 or Varian Liberty 220 respectively).

Plant material analysis

Plant samples were dried overnight in an oven at 70 °C. Subsequently the samples were dried over 3 h at 105 °C for determination of moisture content. Dried material was ground to a fine powder in an electric grinder. Plant material samples (1 g) were digested with 10 mL of concentrated nitric acid in a pressurized vessel lined with Teflon using a computerized microwave system (MDS 2000, CEM, USA). Metal content was determined with AAS spectrometer (Varian SpectrAA 300). The final results were recalculated on a dry weight basis.

All statistical analyses including ANOVA and LSD tests were conducted using STATISTICA software. Sample replications were indicated in the figure legend. A probability of 0.05 or less was considered to be statistically significant.

Results

The stress effects of different doses of EDTA on *Ricinus communis* plants are shown in Figure 2. The highest stress was observed in treatment 2 (EDTA applied in one dose).

Figure 2. *Ricinus communis* plants stressed by different regimes of EDTA dosing (1- control, 2- EDTA in one dose, 3- EDTA in two doses, 4- EDTA in three doses)

The concentration of lead in shoots of transplanted *Ricinus communis* plants are shown in Figure 3.

Based on mass of metal taken up, the most effective treatment was the EDTA applied in one dose, in which case the metal concentrations in shoots of *R. communis* were as follows: lead 5,103 mg/kg d.w.; cadmium 3.14 mg/kg d.w.; and zinc 474 mg/kg d.w.

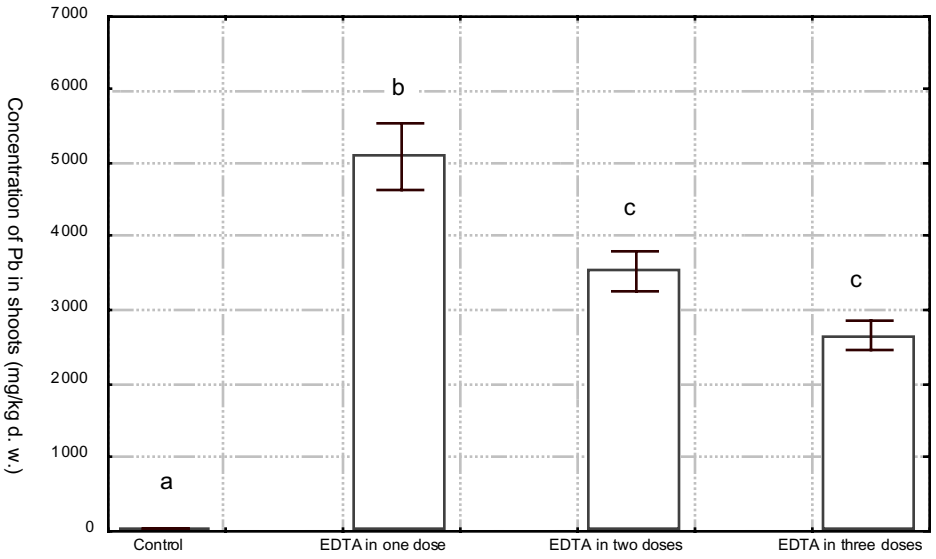

Figure 3. Concentration of Pb (mg/kg d.w.) in shoots of transplanted *Ricinus communis* plants. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

Phytoextraction values also were highest for this treatment. Plant shoots were able to extract 14.21 mg Pb/kg of soil (Figure 4), 0.009 mg Cd/kg of soil and 1.26 mg Zn/kg of soil.

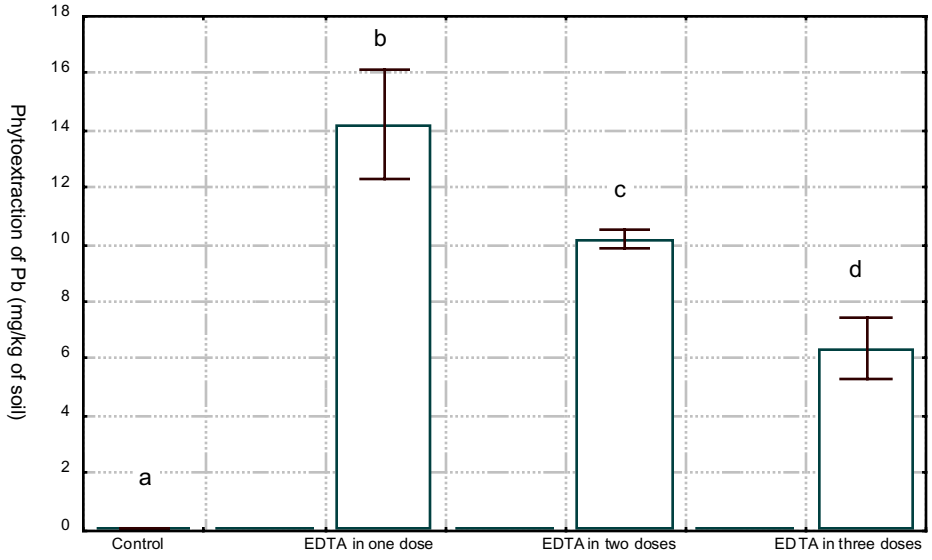

Figure 4. Phytoextraction of Pb (mg/kg of soil) by transplanted *Ricinus communis* plants. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

EDTA applied in one dose was also the most effective treatment for white mustard (directly seeded and transplanted) and sunflower (directly seeded). Lead concentrations in shoots of white mustard and sunflower are presented in Figure 5. Lead content was highest in shoots of directly seeded white mustard (16,500 mg/kg). The same effect was observed with cadmium (11.4 mg/kg) and zinc (2157 mg/kg).

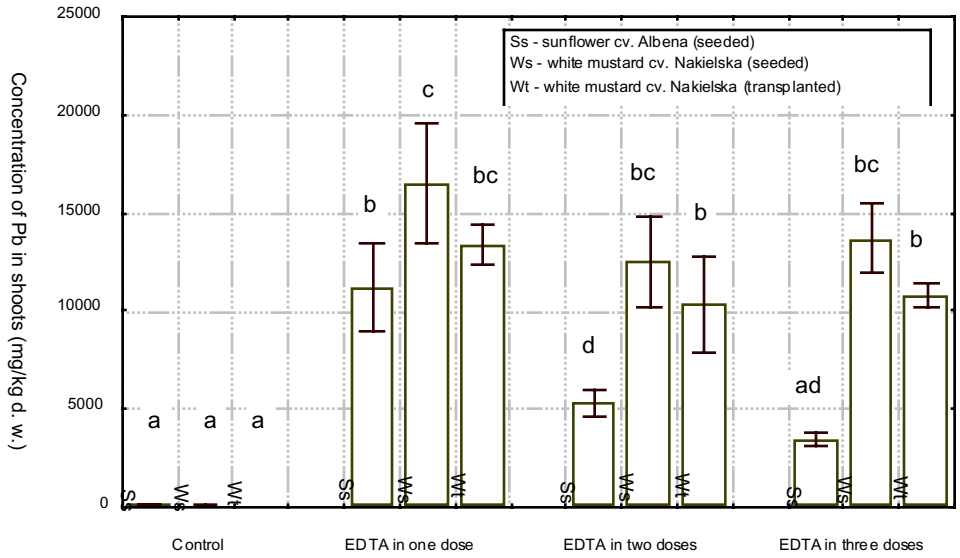

Figure 5. Concentration of Pb (mg/kg d.w.) in shoots of sunflower and white mustard. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

Although lead concentration was higher in shoots of seeded than transplanted white mustard (Figure 5), metal phytoextraction was higher in shoots of transplanted white mustard (26.65 mg/kg of soil) (Figure 6).

The concentration of lead in shoots of sunflower was lower (11,200 mg/kg) than in seeded white mustard. However, the overall lead phytoextraction was better in case of sunflower (27.42 mg/kg of soil) because of its higher biomass production (Figure 6).

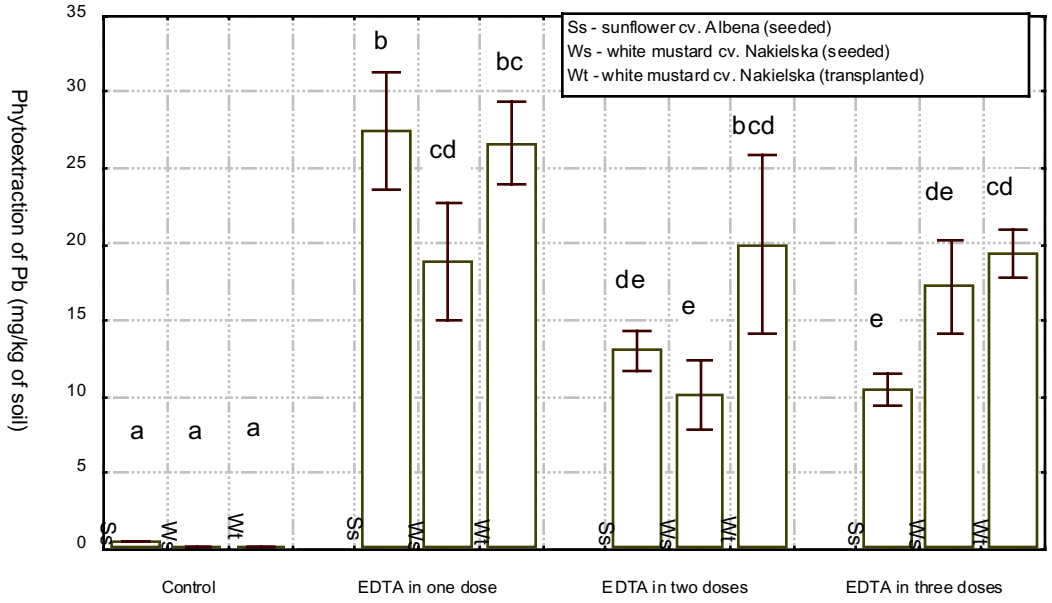

Figure 6. Phytoextraction of Pb (mg/kg of soil) by sunflower and white mustard. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

Concentrations of cadmium and zinc in plant shoots are presented in figures 7 and 8.

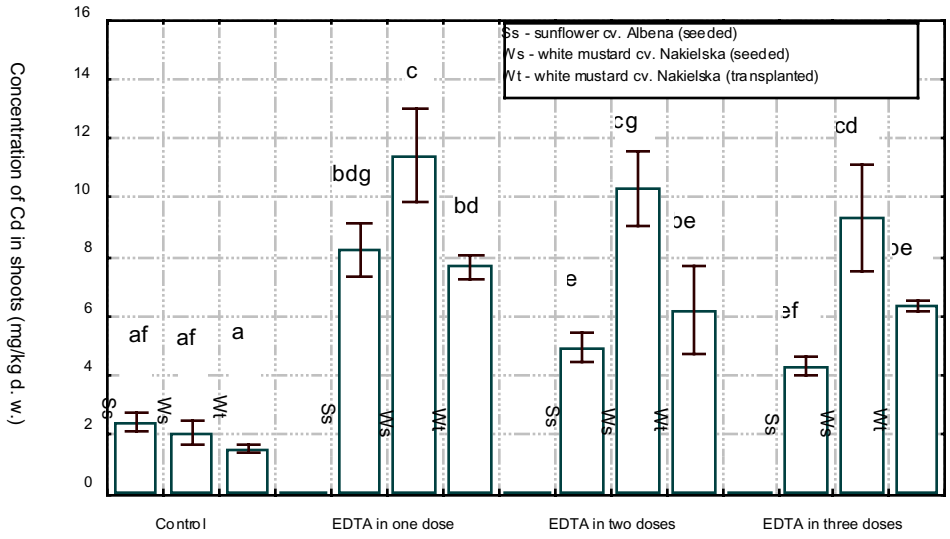

Figure 7. Concentration of Cd (mg/kg d.w.) in shoots of sunflower and white mustard. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

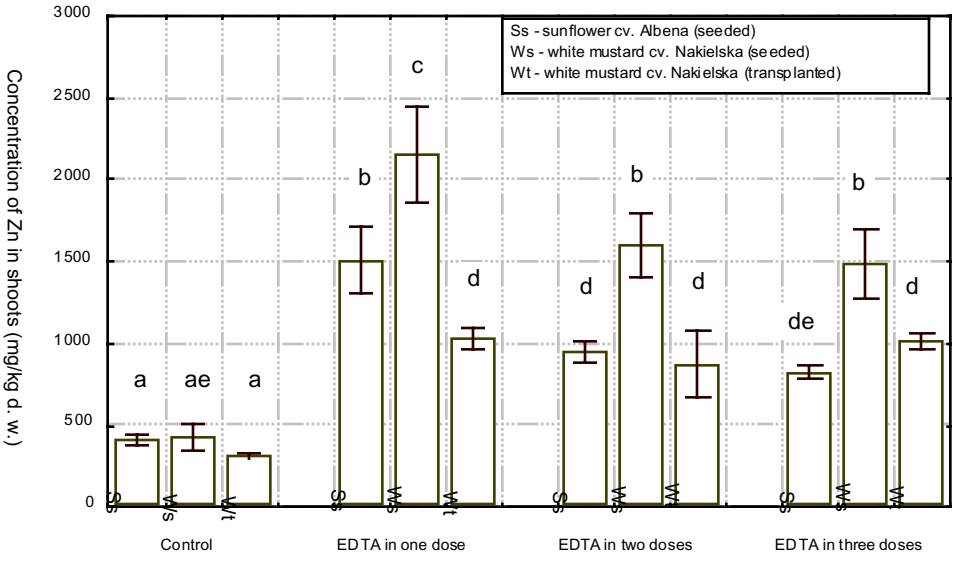

Figure 8. Concentration of Zn (mg/kg d.w.) in shoots of sunflower and white mustard. Values are means – SD (n=5). Means followed by the same letter are not significantly different from each other using the LSD test ($p < 0.05$).

Although cadmium and zinc concentrations in soil were low (Table 1), accumulation of both metals in plant shoots was high.

To quantify phytoextraction efficiency, a phytoextraction coefficient can be used. The phytoextraction coefficient is the ratio of concentrations of a given compound in the plant material to concentrations in the soil. Coefficients for lead are given by Kumar et al. (1995). In experiments conducted to date by the IETU Phytoremediation Team, the phytoextraction coefficient did not exceed the value of 1.5 for lead. For the current Treatability Study the calculated phytoextraction coefficient was much higher and reached mean values: for sunflower cv. Albena for lead 16.74 in the treatment with one dose of EDTA, and 19.94 for transplanted white mustard with the same treatment. The highest lead phytoextraction coefficient (**24.7**) was determined for seeded white mustard with one dose of EDTA. Table 6 shows phytoextraction coefficients for different plant species grown in different soil.

Table 6. Phytoextraction coefficients for different plant species

Source	Plant species	Value
Kumar et al., 1995	*Brassica juncea* cv. 182921	1.70
Huang and Cunnigham, 1996	*Zea mays* cv. Fiesta	4.24
Cooper et al., 1999	*Helianthus annuus* L.	1.29
Agricultural land in Poland (IETU Final Report 1999)	*Sinapis alba* cv. Nakielska	1.50
Firing range in Poland (current study)	*Sinapis alba* cv. Nakielska	17.0-**24.0**

Discussion

In earlier studies (DOE, 2000) it was demonstrated that the application of EDTA in three steps can enhance heavy metal accumulation in plant leaves to a greater extent than the one step treatment. The current study did not support these results (figures 3, 5, 7, 8). It was observed that the most efficient for lead, cadmium and zinc accumulation in sunflower and *Ricinus communis* plant shoots was EDTA and acetic acid applied in one dose. Although in shoots of white mustard higher concentration of the metals were measured, results are not statistically significant (LSD test, $p > 0.05$).

Since the germination and growth of seedlings would be affected by high metal concentration within the soil, the transplanting procedure described by Wu et al. (1999) and Cooper et al. (1999) was compared to the procedure of directly seeded plants. Sunflower (*Helianthus annuus* cv. Albena) and white mustard (*Sinapis alba* cv. Nakielska) were seeded directly into pots with contaminated soil. *Ricinus communis* and white mustard were transplanted into the pots with contaminated soil after growing in clean soil using the procedure of Wu et al., (1999).

The highest concentration of lead was measured in shoots of white mustard seeded directly into the soil (Figure 5). Our results are not in agreement with results Wu et al. (1999), who observed lower concentration of lead in directly seeded corn plants in comparison with transplanted plants. This may be due to differences in plant species.

Biomass production of white mustard, seeded directly into the soil was lower, compared to biomass of transplanted white mustard. For this reason, metal phytoextraction was higher in transplanted white mustard than in white mustard seeded directly into the soil, but these results are not statistically significant (LSD test, $p> 0.05$), except for treatment in which EDTA was applied in two doses (Figure 6). The transplanting procedure at field scale can be time and labor consuming. It seems that economic calculations should be conducted before the transplanting procedure will be used at field scale.

Kumar et al. (1995), in sand-Perlite mixture spiked with 500 mg/kg Pb, observed the phytoextraction coefficient of 1.7 for *Brassica juncea* cv. 182921. A similar phytoextraction coefficient (Table 6) was reported by Cooper et al. (1999) for *Helianthus annuus*, grown in highly contaminated soil (3,989 mg Pb/kg). Huang and Cunningham (1996) observed the higher phytoextraction coefficient for *Zea mays* cv. Fiesta, when grown in soil containing 2500 mg Pb/kg. The current study reports phytoextraction coefficients (17 —24) that are higher than any published values found in the literature.

The unusually high phytoextraction coefficients may result from the mineralogical structure of the target soil, which consists mainly of sand and has weak metal-binding properties.

The high phytoextraction coefficients obtained in this experiment (one dose of EDTA and acetic acid with white mustard and sunflower) suggest that experiments under

field conditions should give promising extraction results of lead from firing range soils. While these phytoextraction values are exciting, it must be stressed that they result from Treatability Studies under laboratory conditions. Field-scale experiments are planned to confirm these results.

Conclusions

1. Among the tested treatments, the most efficient for lead, cadmium and zinc was EDTA and acetic acid applied in one dose.

2. Lead content was highest in shoots of white mustard seeded directly into the soil (16,500 mg/kg).

3. Metal phytoextraction was higher in transplanted white mustard than in white mustard seeded directly into the soil, but these results are statistically significant only for EDTA applied in two doses.

4. Seeded sunflower cv. Albena extracted more lead than seeded white mustard and approximately the same as transplanted mustard for EDTA applied in one dose.

5. High phytoextraction coefficients, between 16.7 and 24.7, were found in all species and treatments.

6. Very high phytoextraction coefficients, if confirmed, should give promising extraction of lead from firing range soil in the future.

7. Phytoextraction continues to be a promising technology for reducing concentrations of lead in surface soils. The results presented here, while promising, emphasize the variability in lead phytoextraction across soil types and plant species, emphasizing the site specific nature of this technology.

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INNOVATIVE APPROACHES TO MERCURY CONTAMINATION IN SOIL

1. Introduction

This report presents current 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 (IETU), Katowice, Poland.

The IETU is evaluating several potential approaches for managing mercury contamination in soil. The technologies addressed in this manuscript include:

- chemical/plant stabilization,

- volatilization.

In order to achieve mercury stabilization in soil, lab-scale experiments were conducted in FY00. Common, inexpensive chemical substances (i.e., sulfur, zeolite, biodecol), which are known for their ability to bind metals, were investigated. The addition of 0.5% granular sulfur to soil resulted in the binding of 78% of water-soluble and exchangeable mercury compounds while the addition of zeolite to soil resulted in the binding of 49% of these fractions. The work completed in FY00 suggests that chemical/plant stabilization holds promise as an *in situ* method for reducing the mobility and bioavailability of mercury in soil. As reported in the results of the FY00 Final report Evaluation of Novel Mercury Remediation Technology , applying 0.5% granular sulfur and planting the site with meadow grass showed promise for stabilizing mercury contaminated soils *in situ*. Two candidate sites in the vicinity of the IETU were identified for experimental work and samples were collected. Analyses of total mercury in soil were carried out. Site No. 2, the Dwory Chemical Plant, was chosen as the experimental site for the project based on the nature and extent of mercury contamination at the site, the willingness of the site to cooperate and the proximity of the site to the IETU.

2. Site identification

The site in question is a chemical facility known as Dwory, where mercury and its compounds are used in multiple manufacturing processes. The Dwory facility is located in Oswiecim (southern Poland) and has been in operation for over 50 years. It is now a well-recognized chemical enterprise in both domestic and international markets. The facility produces about 100 products including: synthetic rubber, synthetic latex, polystyrene, polyvinyl chloride emulsion (PVC E), polyvinyl acetate (PVAc), gaseous and liquid chlorine, sodium hydroxide, sodium hypochlorite and hydrochloric acid.

For several decades the Dwory facility has been using mercury and its compounds in manufacturing processes. Sources of soil contamination include the following technical processes:

- chlor-alkali industries, where metallic mercury is used as an amalgam-cathode,
- acetaldehyde industries, where $HgSO_4$ was used as a catalyst (until 1997), and
- vinyl chloride production, where activated carbon with $HgCl_2$ was used as a˚catalyst (until 1998).

The production of acetaldehyde from acetylene and of vinyl chloride from acetylene were abandoned (in 1997 and 1998 respectively). At present, metallic mercury is used only in the chlor-alkali production processes.

Since 1952, metallic mercury has been used for chlorine and sodium hydroxide production by electrolysis of sodium chloride 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 1993. Since 1994, titanium and fixed parameter anodes have been used. Gaseous chlorine is generated in the electrolysis tank and, after drying, is supplied to other production processes. Sodium amalgamate is transferred from the electrolysis tank to the decomposer where it decomposes into soda lye (45%), hydrogen and mercury in the presence of water. At present the generated hydrogen is utilized as fuel in a˚local power and heating plant. From the decomposer, mercury is pumped to the electrolysis tank. In 2000 the chlorine production volume was approximately 30,000 Mg/yr; metallic mercury consumption was - 17.8 g/Mg chlorine.

The Dwory Chemical Plant is located in the southeastern part of Poland, in the basin of the Sola River - a tributary of the Vistula River and in the drainage area of the upper run of the Vistula River. The facility occupies about 430 ha, which include 336˚ha inside the fence. Buildings cover an area of approximately 38 ha.

2.1. Geology

The majority of the Dwory Chemical Works is located in the western part of the Carpathian Depression. A small part of the facility is included in the southeastern part of the Upper-Silesian Depression.

The following geological formations are present at the site:

- Carboniferous formations — Upper Carbon,
- Tertiary formations — marine Miocene, and
- Quaternary formations.

Carboniferous formations are directly topped with tertiary deposits consisting of marine Miocene formations (torton). These are layers of krakowieckie clays, frequently

limy with sandbanks and layers of chemical deposits (gypsum, anhydrates, etc) of up to 300 m in thickness.

The roof of the Miocene formations, shaped by the Vistula River, is generally flat with a˚moderate, downstream slope. Miocene formations are topped with Quaternary deposits composed of fluvioglacial accumulation formations:

- fluvial - related to the activity of the Vistula River, located within the Vistula terrace up to the datum of 225 m above sea level (ASL),

- glacial - related to glacial activity at the high Vistula terrace above 225 - 230 m ASL.

The thickness of the Quaternary formations is highly variable and depends on local topography and configuration of the Miocene stratum roof.

The high Vistula terrace consists of the following formations (going downwards from ground surface): loesses (silts, sandy silts) and loessy loams 6.0 - 13.5 m thick occur under a thin layer of topsoil typical for the site. Below the silty loams, muds and peats of 1 - 3 m thick occur locally. These formations are underlain by a˚continuous layer of sands, gravels, pebbles and cobbles extending over a Miocene series of clays. The thickness of the gravel and sandy formations is 6 - 15 m.

2.2. Hydrogeological conditions

One aquifer is beneath the chemical plant, which is connected with Quaternary formations, specifically with permeable gravel and sandy layers extending over a˚thick impermeable layer of Miocene clays. The highest point of the basement complex is approximately 228 m ASL. The complex generally slopes 6% toward the northeast.

The filtration coefficient for the aquifer averages $k = 4.6 \times 10^{-4}$ m/s.

The quaternary aquifer is in hydraulic contact with surface waters of the Vistula and Sola rivers. This contact supplies water to the aquifer by infiltration from the rivers at higher water levels.

As a result of intensive groundwater pumping for drinking purposes a large depression of the water table occurred and local directions of water flow have changed. Nevertheless, the Vistula and Sola rivers drain the area during periods of low water levels.

The described Quaternary aquifer, connected with gravel and sandy deposits of an average thickness of 12 m, is a layer of continuous, extensive and of high importance. The aquifer is the major source of groundwater in the Oswiecim area and for several kilometers downstream in the Vistula River Basin. As such, the aquifer requires special protection.

2.3. Site characterization

Three sources emitted mercury to the atmosphere from the facility site: chlorine production using electrolysis, acetaldehyde production from acetylene and coal combustion in the facility s power and heating plant. Since 1998, the sources of mercury emission have been the chlor-alkali industry and thermal power plant. Annual mercury emission volumes from all technological processes are presented in Table 1.

Table 1. Mercury emission volumes [kg/year]

Plant	1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
Chlor-alkali production	45	38	40	50	45	50	42	28	34	38	39
Acetaldehyde production	260	86	90	113	103	114	95	64	-	-	-
Power and heating plant	89	81	75	69	69	74	70	66	56	48	41

To determine surface water contamination, data were used from measurements carried out for the Sola and Vistula rivers up and downstream. Annual median mercury concentrations in these rivers are presented in Table 2.

In Poland the mercury standards for surface water and drinking water are 1.0˚μg/L. Additionally the Polish Environmental Protection Inspection issued guidelines for permissible mercury concentration in groundwater in the following areas:

- protected areas - 0.05 μg/L,
- agricultural land, forest and public use places - 0.3 μg/L, and
- industrial sites - 2 μg/L.

Table 2. Annual median mercury concentrations [μg/L] in the Sola and Vistula rivers

Year	Sola - at confluence of the Vistula River	Vistula - upstream from the facility and Sola River confluence	Vistula - downstream from the facility
1992	0.60	2.50	1.70
1993	3.55	3.70	2.10
1994	1.95	6.00	0.80
1995	n. d.*	n. d.*	n. d.*
1996	0.33	0.90	0.75
1997	0.54	0.21	0.28
1998	0.93	0.89	2.89
1999	0.63	1.46	2.11
2000	0.38	0.51	0.23
2001	0.61	0.72	0.50

n. d.* - not detected (detection limit - 0.05 μg/L)

Both rivers periodically exceeded the standard until 2000.

No mercury was detected in water collected from 19 piezometers installed in the vicinity of the facility in the years 1996-2001. During 1996-2001, mean mercury concentration [μg/L] in water collected from wells located within the facility was as follows:

- 1996 - 0.46 ± 0.13,
- 1997 - 0.49 ± 0.15,
- 1998 - 0.58 ± 0.25,
- 1999 - 0.69 ± 0.20,
- 2000 - 0.48 ± 0.27, and
- 2001 - 0.48 ± 0.06.

Water collected from wells did not exceed the standard and guidelines for groundwater on industrial sites. However, the lack of piezometers close to sites contaminated with mercury does not allow for an explicit statement regarding vertical migration of mercury.

2.4. Mercury concentration in soil

Based on surveys from 1991-1995 of mercury content in topsoil at Dwory Chemical Works, two mercury-contaminated sites were identified among 35 sites investigated°[1]:

- Site 1 - located in the vicinity of acetaldehyde production installation (approximately 12,000 m^2) and

- Site 2 - located in the vicinity of the two cell rooms for chlorine production (approximately 41,000°m^2).

Total mercury concentration in topsoil from measurement points located in sites 1 and 2 ranged from 10.4 to 3,980.0 mg/kg dw. The mean concentration over the contaminated area was 195.6 ± 294.1 mg/kg dw. Total mercury concentration in topsoil from measurement points located in 33 other sites ranged from 0.1 to 8.7 mg/kg dw. The mean concentration over this area was 1.56 ± 2.50 mg/kg dw [1].

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

- mercury emissions to the atmosphere have been reduced by nearly 8 fold in the last ten years,

- mercury concentrations in the upper run of the Vistula River frequently are higher than in the lower run,

- no mercury has been detected in groundwater in the vicinity of the facility.

Data from the long-term environmental monitoring programs shows no mercury contamination in the areas adjacent to the Dwory facility. It appears that mercury-contaminated soils at the Dwory facility site have not contaminated surface water or groundwater. Thus, the contaminated area is limited to the facility site.

3. Chemical and plant stabilization activities

In 2001, a sub-site (155 m x 25 m) was selected from Site 2 located in the vicinity of the electrolysis cell area using X-MET 920 XRF analyzer. Appendix 1 presents the location of the site and the sub-site. The sub-site was characterized by the highest level of topsoil contamination with mercury (i.e., on the level of 2000 mg/kg dw). Due to unseasonable rainfall in March and April, fieldwork began in May. Seventy-five measurement points were established at the sub-site. Topsoil was sampled from these points in May and analyzed for total mercury content. Total mercury content in collected samples ranged from 416 to 39,215 mg/kg dw; mean concentration over this area was 4,421 ± 5,782 mg/kg dw.

The *in situ* capability of a field portable XRF Analyzer is especially attractive for high speed, low cost screening and characterization of a site. Depending on the nature of the contaminant and the soil matrix, the *in situ* method can offer screening quality data with practically no sample preparation at all. To reduce variability, the field technician can mix and composite a sample on the ground before making *in situ* XRF measurements.

The effects of moisture and particle size can be especially pronounced for *in situ* XRF, so quality assurance is especially important. Several samples are prepared by the full protocol (dry, grind, sieve and split the sample) in the field and compared to the result of the *in situ* measurements. To back up field measurements, samples are collected for laboratory analyses.

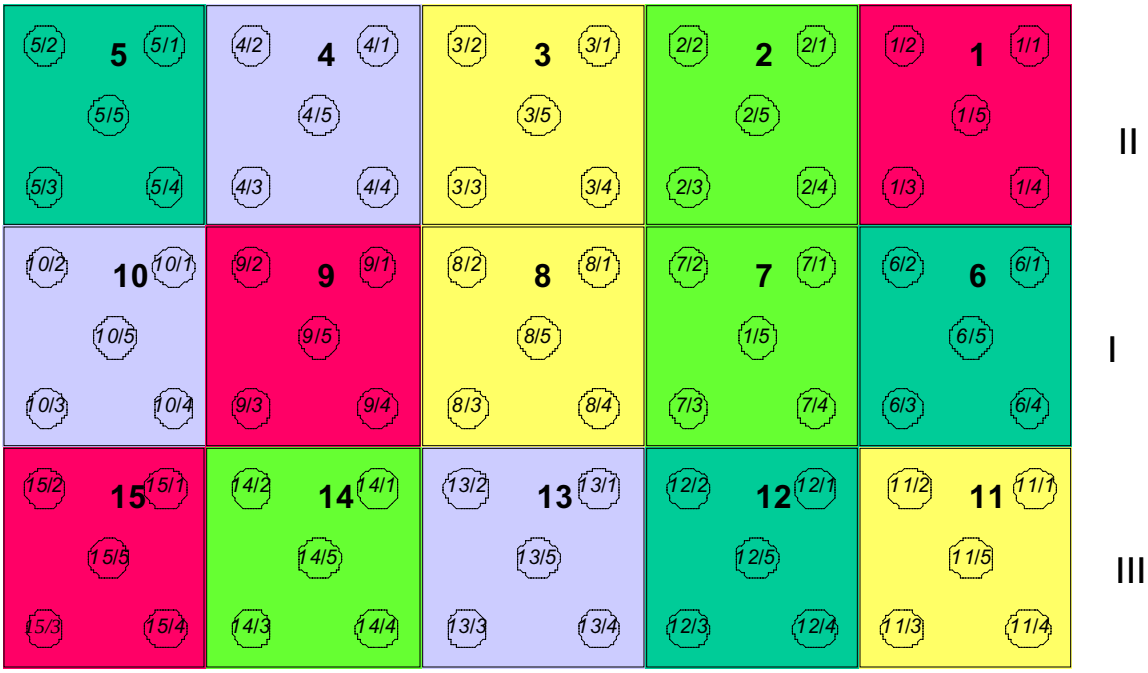
3.1 Experimental plots

Fifteen experimental plots, each 9 m^2 (3 m x 3 m) were delineated within the selected sub-site. Site preparation activities included clearing vegetation and conditioning the soil with a rototiller and mulcher to homogenize mercury levels across plots. Five samples were taken from the soil surface layer (0-20 cm) diagonally across each plot for laboratory chemical analyses.

Plans called for the collection of soil samples from three depths (0-15, 15-30 and 30-45 cm). While preparing plots for chemical and plant stabilization, a rubble heap was found 20-25 cm beneath the entire site. Therefore, site surface characterization samples were collected only in the 0 to 20 cm range.

The experimental site was divided randomly into three blocks: I, II and III (Figure 1). Experimental plots within each block were selected in this same manner. The plots were as follows:

- reference plots (control) without granular sulfur and grass (shown in red),
- experimental plots with granular sulfur (shown in yellow),
- experimental plots with granular sulfur and meadow grass (*Poa pratensis*) (shown in blue),
- experimental plots with meadow grass (*Poa pratensis*) (shown in light green),
- experimental plots with meadow grass (*Poa pratensis*) and willow (*Salix purpurea var. nana*) (shown in dark green).



1 - Plot number
1/1 - Sample number

Figure 1. Plots distribution

3.2. Physical and chemical properties of soil

Surface soils were categorized as sandy loam based on particle size distribution of soil samples within soil classes. Particle size distribution was as follows:

- sand (2.00 - 0.05 mm) 54%,
- loam (0.05 - 0.002 mm) 43%, and
- silt (< 0.002 mm) 3%.

Average values of physical and chemical parameters for soil in the experimental site were as follows:

- cation exchange capacity (CEC) 14.14 cmol+/kg,
  - exchangeable Ca (in CEC) 13.64 cmol+/kg,
  - exchangeable Mg (in CEC) 0.95 cmol+/kg,
  - exchangeable K (in CEC) 0.55 cmol+/kg,
  - exchangeable Na (in CEC) 0.02 cmol+/kg,
- organic carbon 5.30%,

- organic matter 9.14%,
- total nitrogen 0.12%,
- P_2O_5 13.5 mg/100g, and
- K_2O 12.9 mg/100g.

After soil preparation, but before chemical and plant stabilization activities began, pH in KCl and electrical conductance (EC) were determined in samples collected at each plot. The mean values of pH in KCl and EC of soil are given in Table 3.

Table 3. Mean pH in KCl and EC [μS/cm] of soil after soil preparation, but before chemical and plant stabilization (n = 5)

Plot number	pH	EC
1	7.64 ± 0.09	276 ± 41.6
2	7.66 ± 0.06	319 ± 61.2
3	7.61 ± 0.11	334 ± 46.5
4	7.36 ± 0.20	353 ± 53.0
5	6.88 ± 0.33	269 ± 49.5
6	7.52 ± 0.14	252 ± 21.0
7	7.69 ± 0.06	348 ± 62.0
8	7.57 ± 0.14	346 ± 77.5
9	7.23 ± 0.33	316 ± 63.8
10	6.64 ± 0.13	253 ± 65.5
11	7.47 ± 0.26	256 ± 33.3
12	7.59 ± 0.03	296 ± 31.9
13	7.63 ± 0.08	361 ± 93.3
14	7.37 ± 0.08	298 ± 59.4
15	6.67 ± 0.08	296 ± 81.8
Total area	**7.37 ± 0.38**	**305 ± 41.6**

3.3. Mercury concentration in soil before chemical and plant stabilization

Seventy-five soil samples were taken from the plots:

- after soil preparation but before experiment start-up (June),
- six weeks after sulfur addition (July),
- before grass planting (July), and

- at the end of the growing season (October).

The following analyses were conducted on soil samples collected from each plot after soil preparation but before chemical and plant stabilization:

- total mercury,
- water-soluble mercury,
- exchangeable mercury,
- pH, and
- electrical conductance.

Total, water-soluble and exchangeable mercury concentrations in topsoil after soil preparation, but before chemical and plant stabilization of plots are given in Table 4. A˚spatial distribution map for total, water-soluble and exchangeable mercury were developed (See Appendix 2).

Table 4. Mean total, water-soluble and exchangeable mercury concentrations [mg/kg˚dw] in topsoil after soil preparation, but before chemical and plant stabilization (n = 5)

Plot number	Total Hg	Water-soluble Hg	Exchangeable Hg
1	1,403 ± 697	13.63 ± 4.91	180.2 ± 103.8
2	2,556 ± 720	18.16 ± 1.45	359.4 ± 67.6
3	3,650 ± 1,330	20.20 ± 2.15	566.3 ± 196.0
4	5,135 ± 1,641	21.71 ± 2.32	835.6 ± 242.2
5	3,166 ± 1.055	22.77 ± 2.25	629.1 ± 171.1
6	1,792 ± 545	18.62 ± 3.36	222.5 ± 75.5
7	3,956 ± 2,081	20.76 ± 1.98	388.7 ± 111.5
8	3,158 ± 550	17.46 ± 3.78	360.0 ± 27.8
9	5,328 ± 2,572	20.73 ± 2.23	770.8 ± 366.6
10	3,084 ± 1,349	19.00 ± 4.49	690.2 ± 164.7
11	1,644 ± 1,133	12.83 ± 6.06	186.5 ± 145.8
12	3,744 ± 855	17.22 ± 1.69	437.1 ± 94.9
13	3,449 ± 1,135	16.08 ± 2.64	411.7 ± 56.4
14	4,713 ± 2,170	16.84 ± 1.47	630.6 ± 202.8
15	2,478 ± 672	14.74 ± 2.57	622.4 ± 168.1
Total area	**3,284 ± 1,702**	**18.05 ± 4.91**	**486.1 ± 253.8**

SULFUR ADDITION:

7.0 kg of granulated sulfur was added to the soil of each of the appropriate plots. The granulated sulfur was mixed in the soil using a˚rototiller and mulcher to the depth of 15 cm. Calculated sulfur concentration in soil was 0.5% by weight.

3.4. Mercury concentration in soil 6 weeks after sulfur addition

Six weeks after sulfur addition, soil samples were collected from each plot, and soil pH in KCl and EC were determined. The mean values of soil pH are as follows:

- plots without sulfur addition (n=45) - 7.41 ± 0.39, and
- plots with sulfur addition (n=30) - 7.39 ± 0.36.
- The mean values EC [μS/cm] of soil are as follows:
- plots without sulfur addition (n=45) — 298.0 ± 54.6, and
- plots with sulfur addition (n=30) — 323.0 ± 67.1.

No significant differences were observed in pH and EC of soil between plots with and without sulfur addition.

Mean concentrations of water-soluble and exchangeable mercury compounds were determined in topsoil 6 weeks after sulfur addition. The mean values of water-soluble mercury compounds are as follows:

- plots without sulfur addition (n=45) - 18.66 ± 3.96 [mg/kg˚dw], and

- plots with sulfur addition (n=30) - 7.30 ± 2.62 [mg/kg˚dw].

- The mean values of exchangeable mercury compounds are as follows:

- plots without sulfur addition (n=45) -465.8 ± 229.1 [mg/kg˚dw], and

- plots with sulfur addition (n=30) - 303.3 ± 156.3 [mg/kg˚dw].

Nearly 60% of water-soluble and nearly 35% of the exchangeable mercury compounds were reduced 6˚weeks after sulfur addition. Reductions of water-soluble and exchangeable mercury compounds concentrations in topsoil were calculated as the proportion of these compounds in plots with sulfur addition to plots without sulfur addition. Spatial distribution maps for water-soluble and exchangeable mercury compounds were developed (See Appendix 3 and 4).

MEADOW GRASS SOWING:

Based on the manufacturer s recommendation of 10 kg/ha and the experimental area, three hundred grams of meadow grass were planted on each of the appropriate plots. Meadow grass (Poa pratensis) was planted 6 weeks after sulfur addition. On each of three additional plots 9 willows (Salix purpurea var. Nana) were planted.

Figure 2. Experimental plots (note willow seedling in on plot 6)

In July, there were heavy rainfalls the area and the experimental area was flooded. Some of the plots remained under water for several days. The meadow grass was destroyed completely. As soon as the plots were dry, meadow grass was planted again.

Figure 3. Plots after heavy rainfalls

3.5. Mercury concentration in soil 18 weeks after sulfur addition and 12 weeks after planting

In October, 18 weeks after sulfur addition and 12 weeks planting, soil samples were collected from each plot. Soil pH in KCl and electrical conductance were determined for each plot. The mean values of soil pH in KCl and EC of soil are given in Table 5.

Table 5. Mean pH in KCl and EC [µS/cm] of soil 18 weeks after sulfur addition and 12 weeks after planting (n = 15)

Plots	pH	EC
Control	7.15 ± 0.36	193 ± 36.8
Sulfur addition	7.18 ± 0.13	243 ± 37.2
Sulfur addition and meadow grass	7.05 ± 0.23	245 ± 39.8
Meadow grass	7.34 ± 0.18	235 ± 33.1
Meadow grass and willow	7.17 ± 0.28	202 ± 36.9

All data statistical analysis including ANOVA and LSD test were conducted using STATISTICA software. A probability of 0.5 or less was considered to be statistically significant. No statistically significant differences (LSD, $p < 0.05$) were found in soil pH among the control plots and plots treated with granular sulfur and plants (Figure˚4). No significant differences were observed in soil electrical conductance among the control plots and plots treated with granular sulfur and plants.

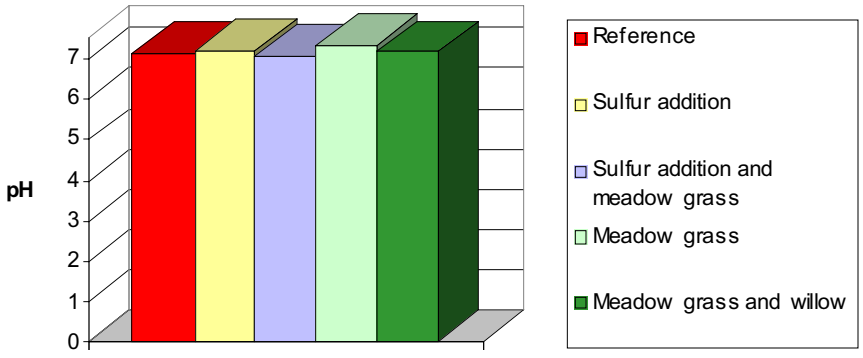


Figure 4. Mean values soil pH

Mean water-soluble and exchangeable mercury compounds concentrations in topsoil (18 weeks after sulfur addition and 12 weeks after planting) are given in Table 6.

Table 6. Mean water-soluble and exchangeable mercury concentrations [mg/kg dw] in topsoil 18 weeks after sulfur addition and 12 weeks after planting (n = 15)

Plots	Water-soluble Hg	Exchangeable Hg
Control	18.02 ± 5.69	489.4 ± 301.1
Sulfur addition	5.92 ± 2.12	212.0 ± 127.3
Sulfur addition and meadow grass	6.02 ± 1.82	348.9 ± 138.6
Meadow grass	16.26 ± 2.26	465.2 ± 164.7
Meadow grass and willow	12.64 ± 2.55	432.0 ± 185.5

Nearly 65 % of water-soluble mercury compounds were converted to more stable forms. Reductions of water soluble and exchangeable mercury compounds concentration in topsoil were calculated as the proportion of these compounds in plots with sulfur addition to reference plots. Statistically significant differences (LSD, $p < 0.05$) in water-soluble mercury compounds were found between control plots, plots treated with granular sulfur and plots stabilized with meadow grass and willow (Figure 5). Spatial distribution maps for water-soluble and exchangeable mercury compounds were developed (See appendices 3 and 4).

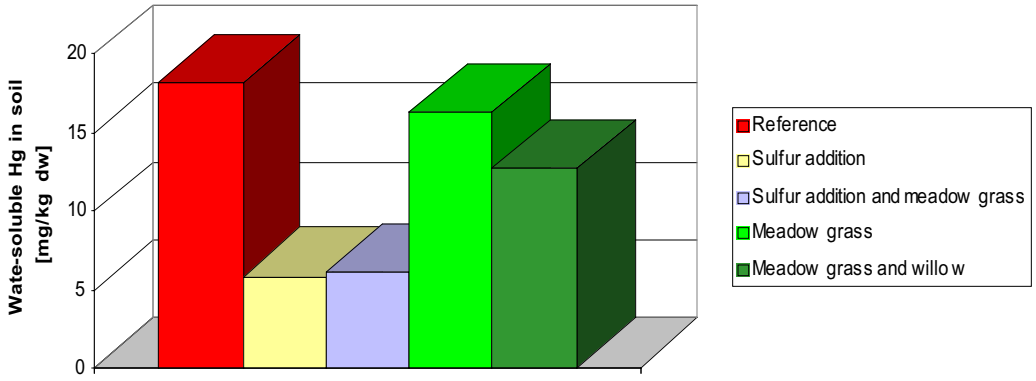


Figure 5. Mean values for water-soluble mercury compounds in soil

Nearly 44% of exchangeable mercury compounds were converted to more stable forms after 18 weeks sulfur addition. No statistically significant differences (LSD, $p < 0.05$) in exchangeable mercury compounds were found between control plots and plots treated with plants (Figure 6).

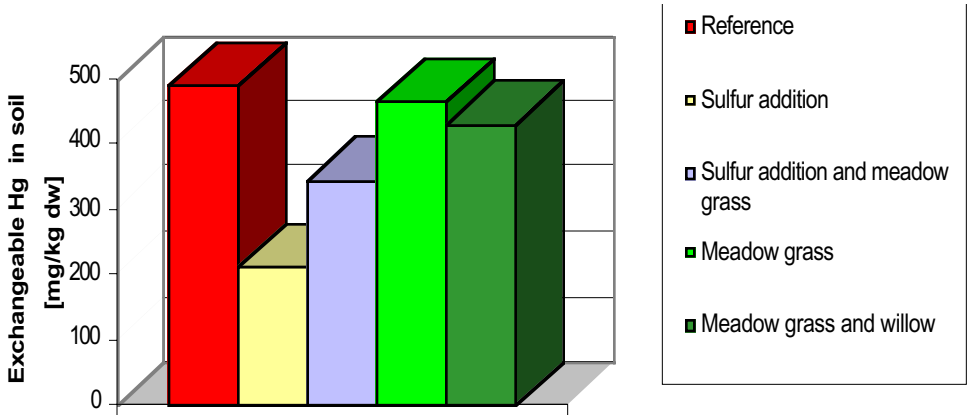


Figure 6. Mean values for exchangeable mercury compounds in soil

Mean values of pH and EC of topsoil at the beginning the experiment, 18 weeks after sulfur addition and 12 weeks after planting are given in Table 7.

Table 7. Mean pH in KCl and EC [µS/cm] of soil at the beginning the experiment, 18 weeks after sulfur addition and 12 weeks after planting (n = 15)

Plots	pH		EC	
	Beginning	18 weeks after	Beginning	18 weeks after
Control	7.18 ± 0.47	7.15 ± 0.36	296 ± 62.1	193 ± 36.8
Sulfur addition	7.55 ± 0.18	7.18 ± 0.13	312 ± 66.1	243 ± 37.2
Sulfur addition and meadow grass	7.21 ± 0.45	7.05 ± 0.23	323 ± 84.2	245 ± 39.8
Meadow grass	7.57 ± 0.16	7.34 ± 0.18	321 ± 60.2	235 ± 33.1
Meadow grass and willow	7.33 ± 0.38	7.17 ± 0.28	272 ± 38.3	202 ± 36.9

No statistically significant differences (LSD, $p < 0.05$) were found in soil pH at the beginning of experiment and 18 weeks after sulfur addition. Small changes in mean values of electrical conductance were seen which may be attributed to natural processes in soil and are not expected to influence these results.

Figures 7 and 8 present the effects of sulfur and plants on water-soluble and exchangeable mercury concentration reductions in soil 18 weeks after sulfur addition and 12 weeks after planting.

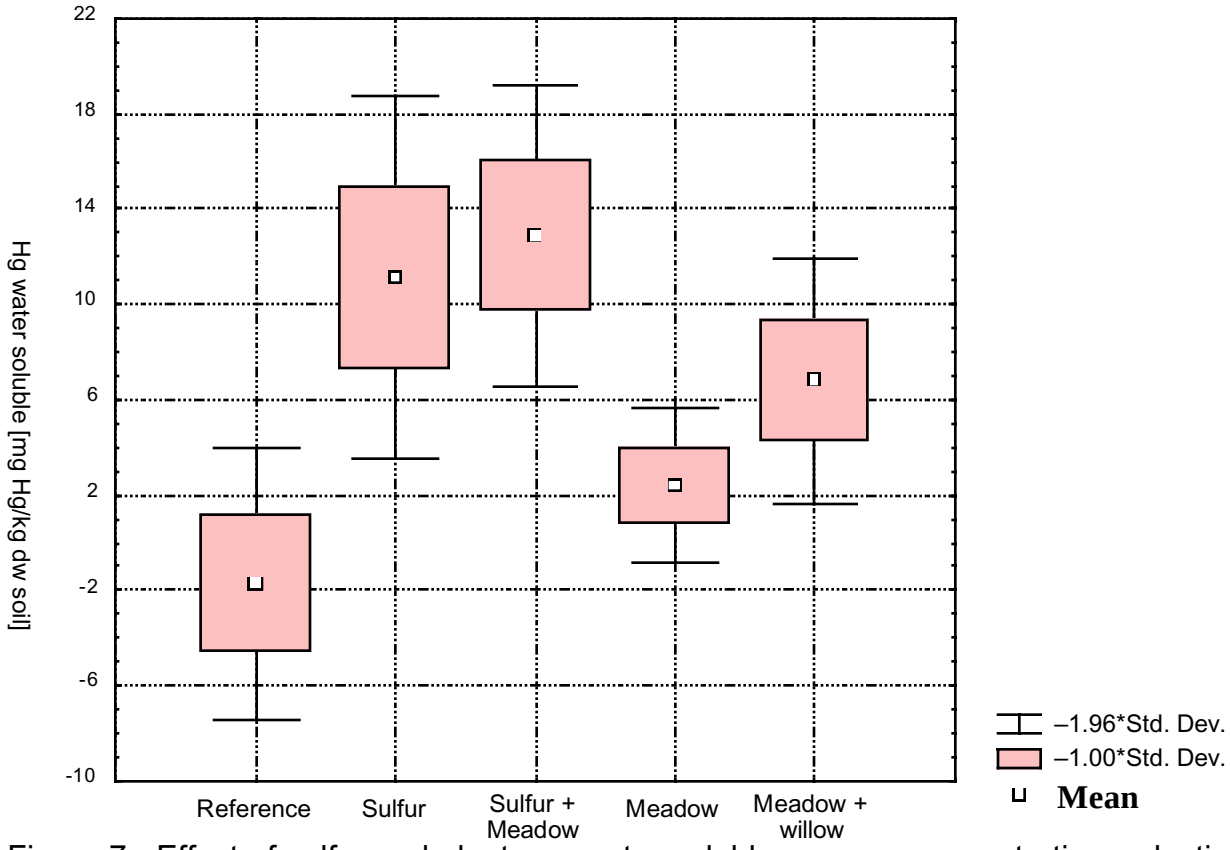


Figure 7. Effect of sulfur and plants on water-soluble mercury concentration reduction in soil 18 weeks after sulfur addition and 12 weeks after planting

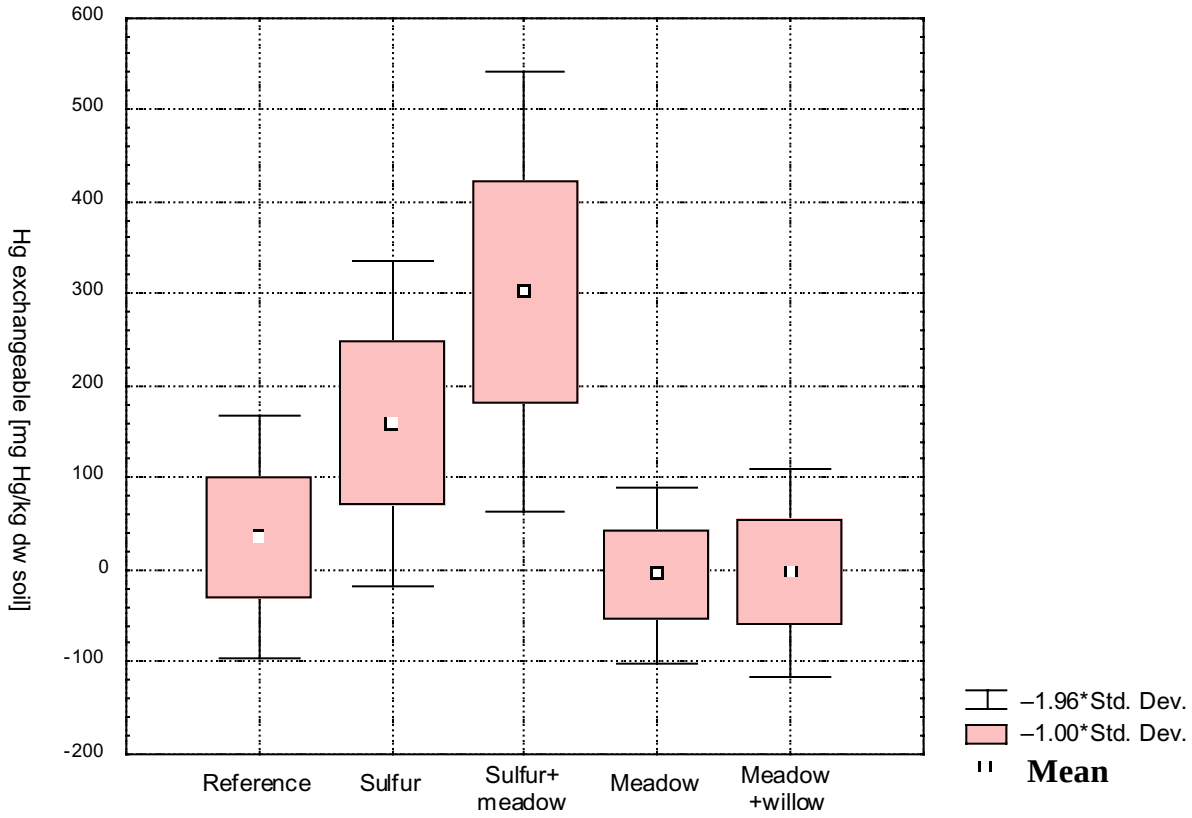


Figure 8. Effect of sulfur and plants on exchangeable mercury concentration reduction in soil 18 weeks after sulfur addition and 12 weeks after planting

3.6. Mercury concentrations in plants

Plant shoot samples were collected from experimental plots. Plant material was rinsed with water. Total mercury concentration was determined by AAS. Results are given in Table 8.

Table 8. Mercury concentrations [mg/kg dw] in plants

Plot number	Meadow grass	Willow
2	31.57	
4	15.22	
5	22.51	30.60
6	12.04	18.90
7	**43.06**	
10	**11.27**	
12	**19.03**	35.37
14	61.69	

3.7. Soil solution

Teflon vacuum cup lysimeters were installed on 12 plots at a depth of 25 cm. Every two weeks, soil solution samples were collected to determine pH, EC, and concentrations of total Hg, Cl^-, NO_3^- and SO_4^{2-}. Unfortunately, in some Teflon vacuum cup lysimeters soil solutions were not collected or were of insufficient volume. The defective lysimeters were replaced, however the volume of soil solution collected from some lysimeters was still insufficient for analysis. Results are presented in Table 9 in Appendix 5.

Only two plots, plot No. 3 (granular sulfur) and No. 7 (meadow grass), provided soil solution samples for the entire sampling period. The following weighted averages for soil solution pH for all sampling periods were obtained:

- plot No. 1 (reference) - 7.44,
- plot No. 2 (meadow grass) - 7.18,
- plot No. 3 (granular sulfur) - 7.41,
- plot No. 5 (meadow grass and willow) - 7.44,
- plot No. 7 (meadow grass) - 7.36,
- plot No. 8 (granular sulfur) - 7.46,
- plot No. 9 (reference) - 7.38,
- plot No. 14 (meadow grass) - 7.26 and
- plot No. 15 (reference) - 7.12.

No significant differences in soil solution pH were observed among the reference plots and plots treated with granular sulfur or plants.

4. Volatilization activities

Pilot lab experiments at the IETU have shown that mercury can be volatilized from soil and collected. In addition the relationship between volatilization of mercury compounds from contaminated soil and temperature has been established. This relationship served as the basis for removing mercury and its compounds from soil by volatilization and subsequent capture of the vapors. The increase of soil surface temperature can be achieved by constructing a lightweight, plastic covered tunnel over the target area. Current year activities included construction of a meso-scale system at the IETU to evaluate the applicability of this approach to actual contaminated soil. The system was built and is undergoing initial tests. Air containing vaporized mercury is drawn at low speed from the enclosure through a˚scrubber to capture mercury. Either wind or solar energy sources can power the fan. The contents of the scrubber will be periodically removed and recycled (Figure 9).

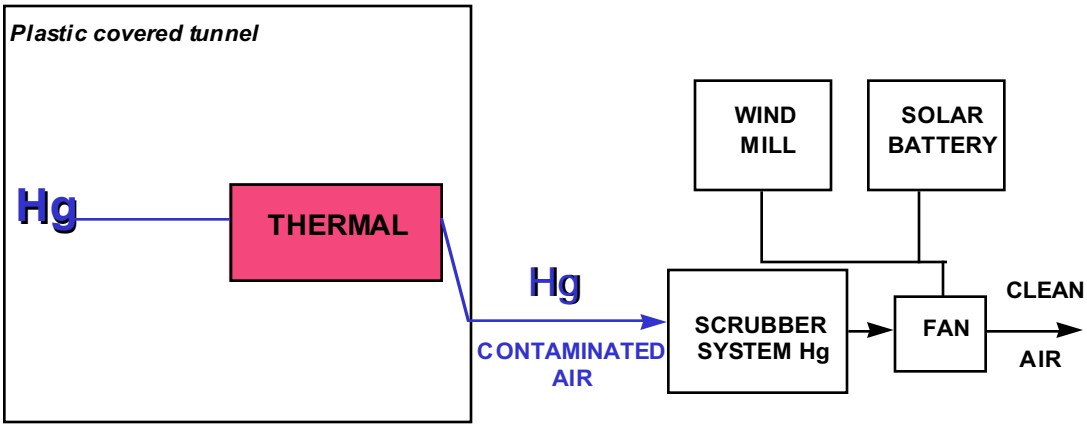


Figure 9. Schematic diagram of a pilot installation for mercury volatilization and capture

To evaluate the feasibility of Hg removal from soil by heating, it was necessary to determine some physical-chemical properties concerning mercury vaporization. Below, data on saturated vapor pressure and vaporization enthalpy of mercury are shown (Poradnik fizyko-chemiczny, WNT, Warszawa, 1974, *Physical-chemical Data Handbook, in Polish pp. 138 - 142).*

Saturated vapor pressure is given by the following equation:

$$\log p = -\frac{A}{T} + B\log + CT + D$$

where:

p - saturated vapor pressure (mm Hg)

T - temperature (K); and

A, *B*, *C*, *D* - empirical constants

for Hg: *A = 3328, B = -0.848, C = 0, D = 10.54.*

In Figure 10 Hg saturated vapor pressure for temperatures in the range of 0 - 100 ^{o}C (273 - 373 K) is shown. Corresponding mercury concentrations in air at 1 Atm are given in Figure 11.

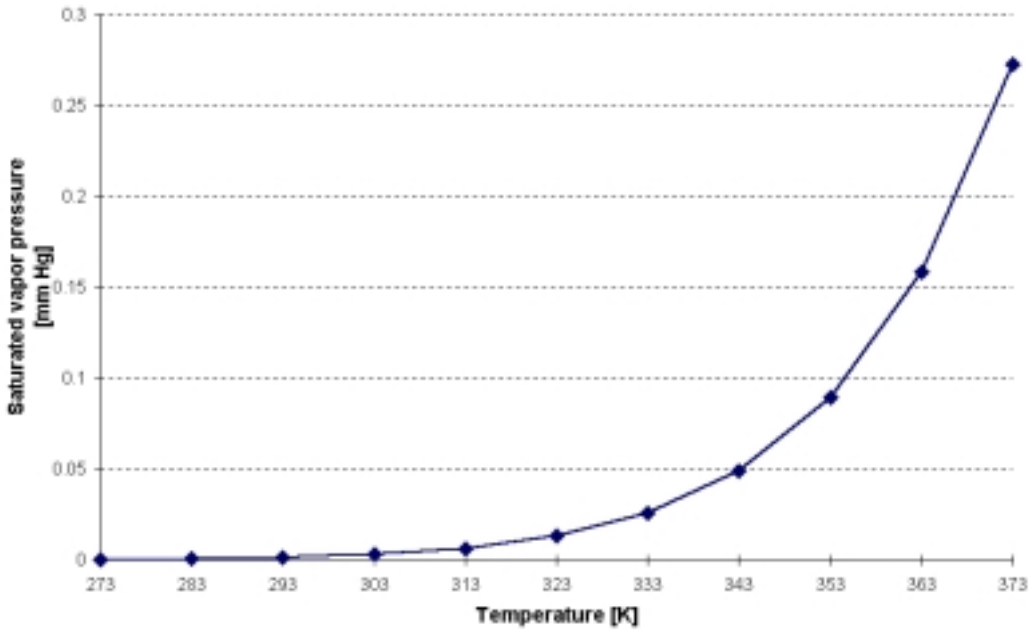


Figure 10. Mercury vapor pressure vs. temperature

Enthalpy of vaporization is given by the following equations:

$$L_p^{\,0} = RT^2 \frac{d\ln p}{dt}$$

$$\frac{d\ln p}{dt} = \frac{A}{T^2} + \frac{B}{\ln 10\, T} + C$$

where:

$L_p^{\,0}$ - enthalpy of vaporization, cal/mole

R - gaseous constant, 1.98 cal/K/mole

T - temperature (K)

p - saturated vapor pressure (mm Hg)

ln - natural logarithm and

A, *B*, *C*, *D* - empirical constants

for Hg: *A = 3328, B = -0.848, C = 0, D = 10.54*.

Pertinent calculations show that in the temperature range 273 - 373 K the vaporization enthalpy of mercury is fairly constant and equal to 73 cal/g.

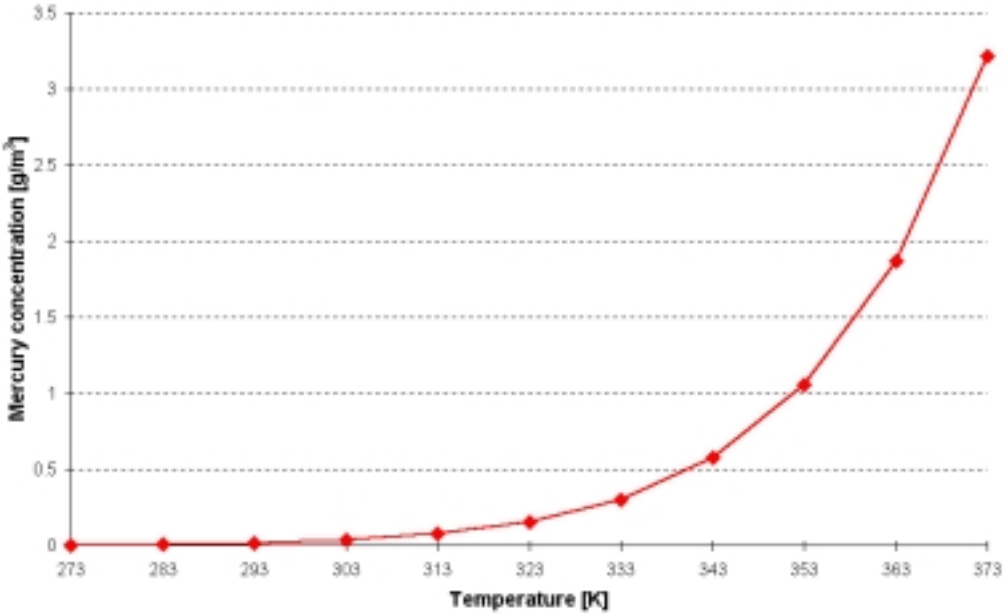


Figure 11. Mercury concentration in Hg saturated air at 1 Atm.

4.1. Methods of increasing soil temperature

When cultivating vegetables and in horticulture, air temperature usually is controlled through the use of greenhouses, hotbeds and plastic covered tunnels. Features of these constructions include permeability for short-wave solar radiation, retention of long-wave thermal radiation and low heat conductivity. Reduced air movement and minimal rainwater infiltration into soil also increases temperature in the enclosed areas. Due to relatively low costs of plastic covered tunnels as well as their technical, construction and application values, analyses were focused on increasing air temperature using such structures.

Greenhouse effectiveness is determined by several factors:

- Intensity of insolation, which depends on latitude, season, time of day, cloudiness and atmospheric obscurity

- Physical properties of the target soil, such as:

- Albedo, i.e., reflected as incident radiation ratio, which refers to direct short-wave radiation, dispersed radiation and long-wave thermal radiation;

- Heat capacity and thermal conductivity connected with soil type and moisture content; and

- Nature and extent of plant coverage.

Physical properties of plastic used for tunnel construction, such as:

Transparency (transmission) and albedo with regard to scattered and direct short-wave solar radiation, and

Heat conductivity coefficient. For transparent sheeting, 0.10 mm thick, the coefficient varies from 0.15 to 0.22 W/m ^{o}C [2]. This determines the greenhouse effect (i.e., heat accumulation in plastic covered tunnels) both in daily and seasonal time frames. Transmission value and albedo of plastic covers depends on the azimuth and sun height above the horizon and, consequently, on the shape of the tunnel and its geographic orientation.

The amount of energy penetrating the soil under the plastic cover can be expressed by the following, general equation:

$$Q_g = Q_k \cdot t_f \cdot (1 - \alpha_g) \cdot (1 + \alpha_g \cdot \alpha_f)$$

where:

Q_g - intensity of radiation penetrating the soil under the plastic sheet,

Q_k - intensity of direct and dispersed solar radiation,

t_f - plastic transparency coefficient,

α_f - albedo of plastic cover, and

α_g - albedo of soil.

Based on a literature survey, plastic covered tunnels usually are constructed of 0.025 - 0.29 mm thick polyethylene (PE), polypropylene (PP) or ethylenovinyloacetone (EVA). To increase soil temperature a single, double or triple layered cover is used. Compared to an open field, soil temperature was increased 7.5 - 8.0 ^{o}C at the soil depth of 10 cm [3, 4, 5]. Such an increase was obtained in a moderate climate, in plastic covered tunnels used for vegetable crop production. It can be expected however, that the temperature of soil without a vegetative cover may be greater.

4.2. Field experiment

The aim of this experiment was to remove mercury and its compounds from soil using thermal methods and to assess the efficiency of this process under field conditions.

4.2.1. Selection of impermeable layers

In order to construct a containment system for field experiments, it was necessary to identify substances that could be used to contain the mercury-contaminated soil. A lab-scale experiment was completed to identify substances, which effectively inhibit mercury penetration in the soil profile. These substances could serve as mercury impermeable layers four larger scale experiment. The following substances were tested: dolomite 1, dolomite 2, dolomite 3, clay and floatation tailings (FT). For experimental purposes 6 columns were prepared: 5 filled with 7 cm of different mercury impermeable materials covered with 30 cm of mercury contaminated soil (535 mg/kg dw), and one control column filled with contaminated soil. Water was added to the lysimeters in defined time intervals and volumes so as to produce a continuous drip from the columns. Soil effluent samples were collected every other day for eighteen days from each column. The most effective impermeable material was chosen based on mercury concentration in effluent. Data on mercury concentration in effluent are given in Table 10.

Table 10. Mercury concentration in effluent [μg/L]

Time of sampling [day]	Soil +dolomite 1	Soil +dolomite 2	Soil +dolomite 3	Soil + clay	Soil + floatation tailings (FT)	Soil
2	0.32	0.22	0.35	0.41	0.25	2.23
4	0.36	0.25	0.28	0.16	0.15	6.38
6	0.38	0.14	0.28	0.38	0.28	2.79
8	0.51	0.18	0.30	0.30	0.33	3.69
10	0.56	0.21	0.36	0.29	0.36	1.81
12	0.37	0.18	0.18	0.21	0.39	1.42
14	0.18	0.15	0.32	0.22	0.43	1.06
16	0.18	0.16	0.26	0.21	0.33	0.95
18	0.17	0.13	0.22	0.19	0.36	0.91

Mercury load washed out from soil at different impermeable layers was calculated on the basis of mercury concentration in effluent and volume (Figure 12).

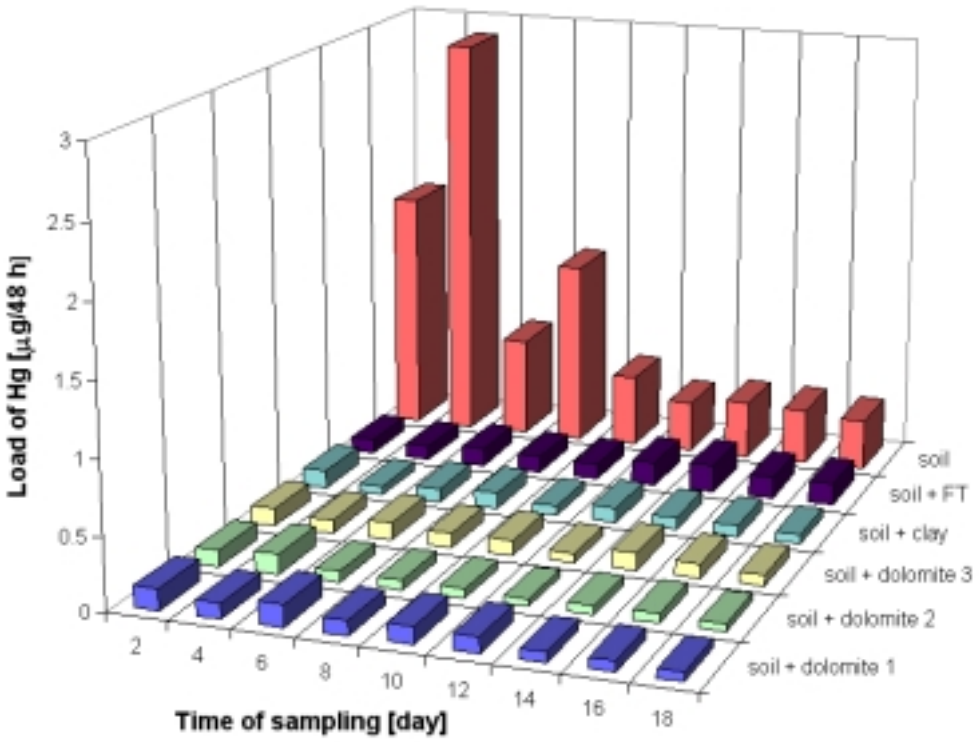


Figure 12. Load of mercury by 48 hours by layer type

Data on mercury content in soil solutions and the calculated loads indicate that under extreme conditions all applied layers showed high impermeability. Based on cost and availability clay was used for fieldwork.

Soil preparation. Approximately 6 - 8 Mg of soil were collected from the surface (0—30˚cmdepth) of the Dwory Chemical Plant site (near the experimental plots) for mercury volatilization studies. Soil was collected from contaminated areas (previously analyzed with the XMET 920, XRF Analyzer) within the selected field, combined into one sample and transported to the IETU where it was homogenized. This soil was used for laboratory experiments on thermal volatilization of mercury and its compounds from soil.

4.2.2. Construction of tunnel

Soil surface temperature increases were studied by constructing a light, plastic, covered tunnel over the target area.

The following types of plastic sheeting, produced in Poland, were used for the experiment:

translucent polyethylene sheeting (PE), 0.155 mm thick, and

translucent polypropylene sheeting (PP), 0.025 mm thick.

Tunnel shape and location.

The tunnel is pyramidal in cross section with a rectangular base and a side slope of 45 ^{o}. It is oriented with its longitudinal axis perpendicular to East and West at real noon on the 23rd of June.

Figure 13. View of experimental installation

The translucent 0.155 mm thick PE was used to construct a walled tunnel of the following dimensions:

- height h = 0.7 m,
- width s = 1.4 m,
- length d = 3.0 m,
- volume V = 1.47m^3,
- base surface area - 4.2m^2, and
- sheeting area - 5.94m^2.

One series of measurements was performed 30 July 2001. Plastic sheeting was inclined at angle of 60^{o}, 50^{o} and 40^{o} from horizontal. The measurements were carried out during cloudless sky and moderate turbidity. The results are summarized below:

Mean time	h_s	X	I_f (W/m^2)	I_o (W/m^2)	t_f	α_f
11^{37}	55.6	64.4	608.8	813.4	0.748	0.222
12^{01}	57.2	72.8	619.3	824.8	0,751	0.219
12^{21}	58.0	82.0	627.9	825.4	0.761	0.209

where:

h_s - sun elevation (degrees above horizon),

X - angle of frame plastic to the horizontal surface,

I_f - intensity of solar radiation behind the frame plastic sheet,

I_o - intensity of solar radiation in front of the frame plastic sheet,

W/m^2 - heat conductivity coefficient,

t_f - plastic transparency coefficient, and

α_f - plastic albedo.

Values of plastic albedo (α_f) were computed assuming an absorption coefficient of $\alpha_f^{\circ} = {}^{\circ}0.03$ according to literature data [5].

Measurement results showed that the differences between both values (t_f, and α_f) were rather small, however increasing the plastic albedo values resulted in decreased X-values.

These measurements were confined to a narrow range of solar incidence angles (X = 64^o - 82^o) and relatively high intensity of solar radiation. Therefore, measurements will be continued under lower values of solar incidence angles reaching the plastic surface.

4.3. Chemical and low-thermal volatilization

A lab-scale experiment was completed to identify the chemical substance, which most effectively volatilizes mercury and its compounds from soil. The volatility of defined mercury species occurring in soil decreases in the following order:

$$Hg^0 \approx (CH_3)_2Hg >>> CH_3HgCl > Hg(OH)_2 > CH_3HgOH > HgCl_2.$$

Two catalysts ($FeCl_2$ x $4H_2O$ and CaO) were added to contaminated soil and the effect on soil temperature and amount of catalyst were examined to determine the percentage of mercury volatilized [6,7]. Contaminated soil was heated to 40-100 ^{o}C to examine the relationship between heating temperature and mercury volatilization percentage versus time. Amounts of catalyst added to soil were 1, 10 and 100 times

greater than the mercury concentration in contaminated soil. Mean mercury concentration in soil was - 2,900 mg/kg dw. Comparisons of amounts of catalyst added to soil vs. temperature are presented on Figure 14 - 17.

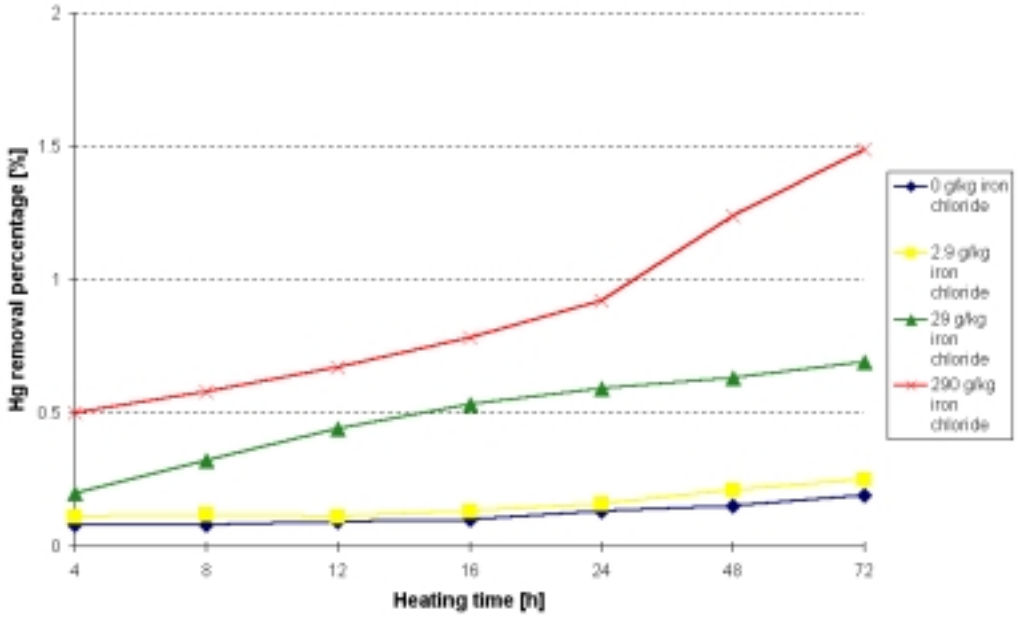


Figure 14. Relationship between soil temperature and mercury volatilization by $FeCl_2$ catalyst amount (heated to 40 ^{o}C)

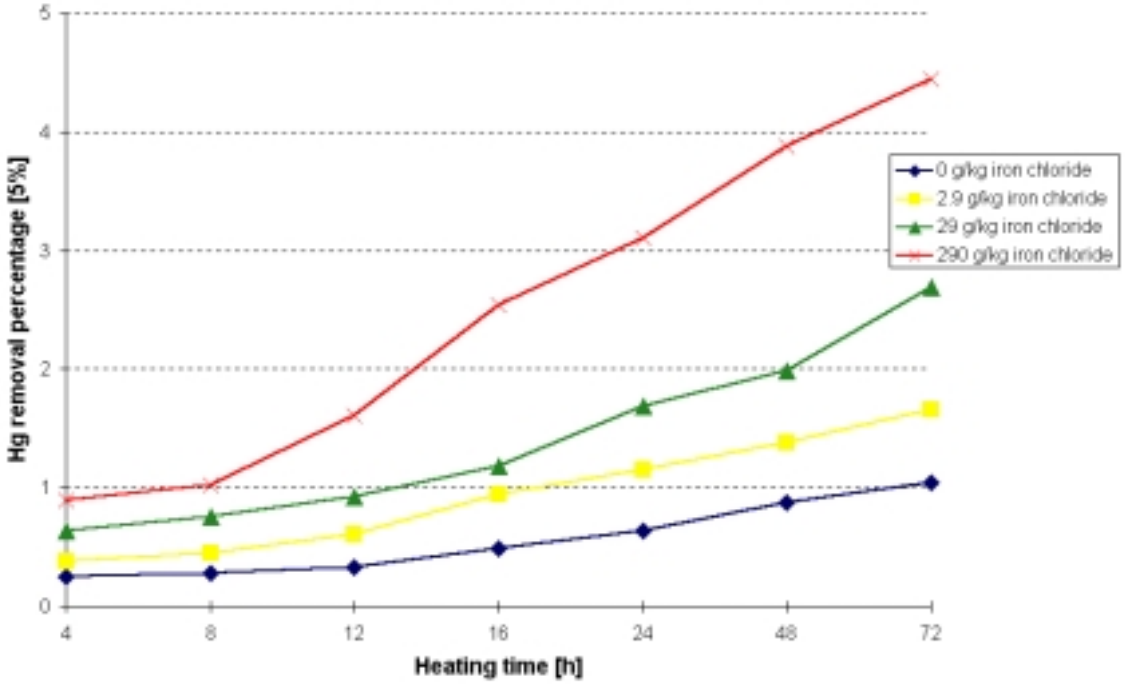


Figure 15. Relationship between soil temperature and mercury volatilization by $FeCl_2$ catalyst amount (heated to 60 ^oC)

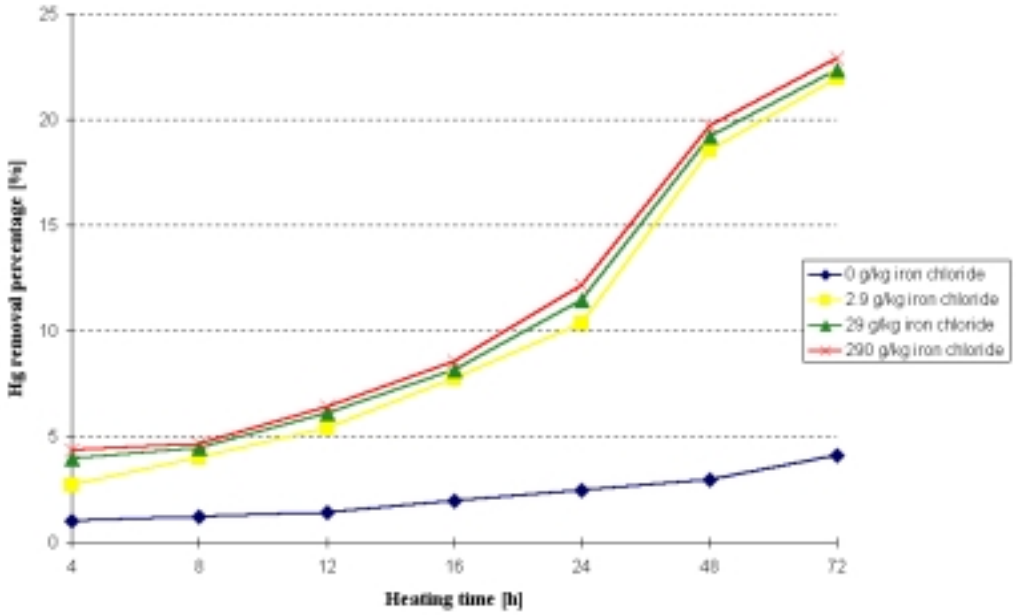


Figure 16. Relationship between soil temperature and mercury volatilization by $FeCl_2$ catalyst amount (heated to 80 ^oC)

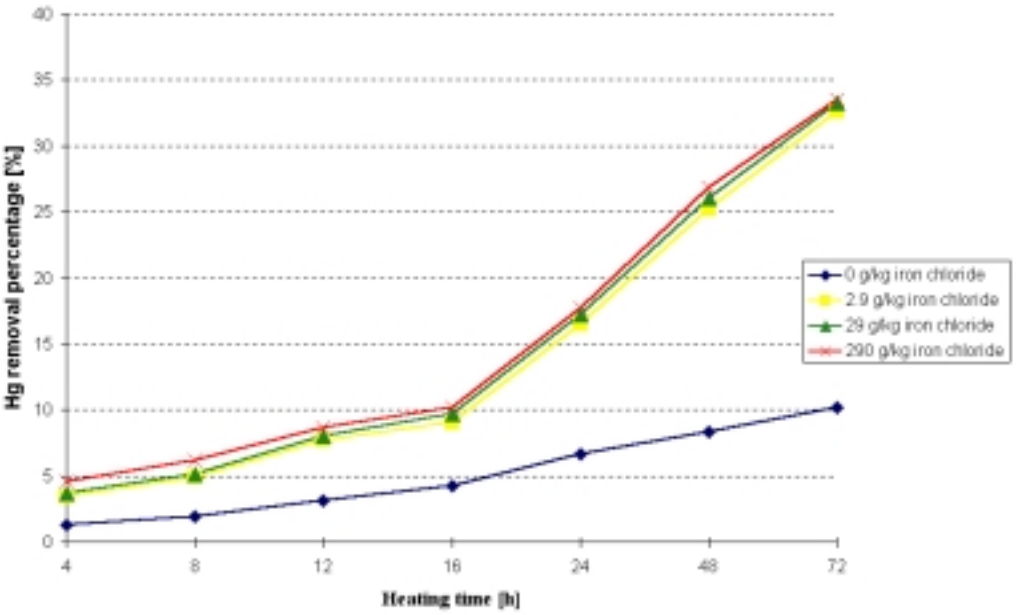


Figure 17. Relationship between soil temperature and mercury volatilization by $FeCl_2$ catalyst amount (heated to 100 ^oC)

A similar relationship between heating temperature and mercury volatilization percentage versus time were observed for calcium oxide addition. In temperatures of 80 ^oC and above, the amount of catalyst added had no effects on mercury volatilization

from soil. However, even low concentrations of catalyst substantially increased volatilization.

5. Summary

Data comparisons of mean pH, EC, water-soluble and of exchangeable mercury compounds concentrations in topsoil at the beginning of experiment, 18 weeks after sulfur addition and 12 weeks after planting (Table 7 and figures 7 and 8) were observed:

- no significant differences in soil pH,
- small changes in mean values of electrical conductance were seen which may be attributed to natural processes in soil and are not expected to influence these results,
- addition of granular sulfur to soil resulted in significant decreases of water-soluble (nearly 65%) and exchangeable (nearly 44%) mercury concentrations in topsoil, and
- in the first growing season no plant stabilization was observed, which may be due to slow growth and development of newly introduced plant species resulting due to flooding.

Typical concentrations of mercury in plants growing around chlor-alkali complexes do not exceed 10 mg/kg d.w. [8,9,10], whereas much higher concentrations of Hg in plant shoots were observed in the current study. The mean total mercury concentration in soil used in the present study was 3,284 mg/kg d.w. (Table 4). It is suggested that high Hg concentrations observed in meadow grass and willow probably are connected with extremely high concentrations of Hg in soil.

Evaluations of solar radiation and air temperature in Poland suggest that solar heat may be used as a source for mercury volatilization from contaminated soil.

In temperatures higher than 80 ^{o}C mercury volatilization is not influenced by differences in catalyst amount.

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