# Soil microbiological damages by overliming[1]

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**Abstract**

Incubation studies were conducted to determine the influence of hydrophobised CaO added to the soil on the number of micro-organisms and their activity. Soil that was contaminated with diesel fuel was mixed with hydrophobised CaO in a ratio of 10:1 in order to immobilise the pollutant. This procedure is an usual method for the decontamination of polluted soils. The deliberate lime surplus caused an increase of soil pH with a maximum value of pH 12.6. The high pH values decreased only very slowly within time. The treatment caused a decrease of the number of bacteria by about 99 %. The population of actinomycetes and fungi was destroyed completely. The incubation study showed that actinomycetes and fungi were not able to recolonise the treated soil. The microbial activity reduced by the application of hydrophobised CaO recovered only incompletely.

Keywords: Liming, immobilisation, DCR-treatment, soil microbiolology, diesel fuel

**Schäden der Boden-Mikrobiologie durch Überkalkung**

In Laborversuchen wurden die Auswirkungen einer Bodenbehandlung mit hydrophobiertem CaO auf die Anzahl und Aktivität von Boden-Mikroorganismen untersucht. Ein mit Dieselkraftstoff kontaminierter Boden wurde mit dem Ziel der Schadstoff-Immobilisierung im Verhältnis 10:1 mit hydrophobiertem CaO gemischt. Diese Methode entspricht einem in der Bodensanierung üblichen Verfahren. Die bewußte Überkalkung verursachte einen Anstieg des pH-Wertes bis auf pH 12,6. Der hohe pH-Wert sank im Verlauf der Zeit nur sehr langsam ab. Die Behandlung löste einen Rückgang der bakteriellen Besiedelung um 99 % aus. Die Populationen von Actinomyceten und Pilze wurden sogar vollständig zerstört. Die Laborversuche zeigten aber auch, daß Actinomyceten und Pilze den behandelten Boden nicht erneut besiedeln konnten. Die mikrobielle Aktivität wurde durch die Zugabe von hydrophobiertem CaO stark reduziert und konnte nur unvollständig wiederhergestellt werden.

Schlüsselworte: Kalkung, Schadstoff-Immobilisierung, DCR-Behandlung, Bodenmikrobiologie, Diesel

## Introduction

Liming of agricultural soils is a compromise between soil structural and plant nutritional aspects. The first requiring a sufficient flocculation of clay minerals which is especially important on heavier soils and which depends on the calcium saturation, the latter preferring lower pH values for optimum conditions especially of micro-nutrients. A multitude of methods to treat contaminated soils exist (DVWK, 1997). Contaminated soils are e.g. treated with a surplus of lime as it reduces the solubility of heavy metals and their bio-availability. Contamination of soils with mineral oil hydrocarbons is an ubiquitous problem and caustic lime (CaO) hydrophobised with stearic acid was used to immobilise organic contaminants, especially mineral oil hydrocarbons (Bölsing, 1988).

Especially in the 70s and 80s soils contaminated by mineral oil hydrocarbons were occasionally treated with hydrophobised caustic lime (CaO) in order to immobilise the pollutant. This method was mainly practised in Europe and called the DCR[3]-method (Stoeven, 1999). Contaminated soils were excavated and subsequently mixed with hydrophobised caustic lime in a ratio of 10:1. The idea was to prevent organic and inorganic pollutants from leaching by embedding them with limestone ($CaCO_3$).

The treatment should minimise the environmental risks with view to the health of humans and irreversible environmental damages for a long time. The treated soil was usually compacted and disposed in land fills or used in road construction (Gerschler, 1981). But the soil will remain contaminated and the time span of immobilisation of hydrocarbons is yet not known.

The present study presents the results of microbial experiments conducted on a sandy soil contaminated with diesel fuel and treated with hydrophobised CaO. The aim of these investigations was to evaluate the effect of CaO-treatment on the number of micro-organisms and microbial activity as it provides information of potential risks for the environment, if these waste are recycled.

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1 in memoriam Dr. Klaus Grabbe

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3 DCR = dispersion by chemical reaction

## Materials and methods

Sandy soil (82.8 % sand, 12.4 % silt, 4.8 % clay, organic carbon < 0.01 %) was contaminated with 2 % (W/W dry matter) diesel fuel and subsequently mixed with 10 % hydrophobised CaO (W/W dry matter). Hydrophobised CaO was prepared by spraying 1-2 % (W/W) stearic acid evenly onto finely ground CaO (producer: FELS WERKE GmbH, Goslar, Germany).

The hydrophobic character of the CaO is necessary to facilitate contact between CaO and hydrocarbons. Additionally the stearic acid retards the reaction of CaO with soil water and atmospheric $CO_2$ to prevent excessive warming (Bölsing, 1988).

In this study the unpolluted soil material is further referred to as "sandy soil", the diesel fuel polluted soil as "sand substrate" and the diesel fuel polluted soil after CaO-treatment as "CaO substrate".

The pH of substrates was measured by means of a pH meter with glass-electrode in 1:2.5 suspension with 0.01 M $CaCl_2$.

The number of heterotrophic aerobic and anaerobic bacteria, actinomycetes and fungi were determined by the spread plate technique. Micro-organisms were collected in 90 ml of a sterile 0.1 % tetrasodium pyrophosphate solution which was added to 10 g of substrate and 5 sterile glass beads (Ø 3 mm) in a 250 ml SCHOTTGLAS flask. The samples were shaken for 10 min at 100 rpm at 20 °C. Coarse particles settled after 10 min; then the before supernatant was decanted. The supernatant was diluted with physiological sodium chloride solution (0.9 % NaCl) and appropriate agar plates were inocculated. For the determination of the populations of aerobic and anaerobic bacteria "Standard I-Nutrient-Bouillon" (MERCK, Darmstadt, Germany) was used 50 % concentrated and solidified by 20 g $l^{-1}$ agar agar. The plates were incubated in an oxygen free atmosphere using "Anaerocult-A-packs" (MERCK) which were prepared in special vessels in order to determine the number of anaerobic bacteria. The inoculated plates were incubated for 7 days at 20 ± 2 °C in the dark. Actinomycetes were enumerated using the method following Drews (1983). Fungi were determined with "Wuerze-Bouillon" (MERCK) 50 % concentrated, amended with 0.03 mg rose bengal and solidified by 20 g $l^{-1}$ agar agar. Plates were incubated for 7 days in the dark. The counting of the formed colonies (CFU[4]) was carried out after incubation.

The density of hydrocarbon degrading micro-organisms (HDM[4]) was estimated using the most probable number (MPN[4]) enumeration according to Werner (1979) which uses diesel fuel as the sole carbon and energy source. The tubes were incubated for 4 weeks at 20 ± 2 °C in the dark.

[4] CFU = colony forming units, HDM = hydrocarbon degrading micro-organisms, MPN = most probable number

The different substrates were incubated in vessels in order to detect long-term effects of CaO treatment to soil micro-biology. Each 10 kg of substrates were levelled at 60 % of the maximum water holding capacity and kept at 15 °C in the dark. The substrates were mixed every 3 days to enable gas exchange.

Microbial activity was measured by means of the oxygen demand using the Sapromat D12 equipment (VOITH GmbH Heidenheim, Germany). The dehydrogenase activity was estimated using the method of Thalmann (1968) modified by Malkomes (1991).

Correlation analysis was performed to show relations between pH value of the substrate, microbial number and microbial activity.

## Results

### *pH*

The initial pH value of the sandy soil was 7.6. Addition of 2 % diesel fuel caused a slight increase in pH of 0.1. Addition of 10 % hydrophobised CaO yielded pH values up to 12.6. This effect was observed in the unpolluted and polluted soil substrates.

The pH of the CaO substrate decreased from 12.6 to 7.9 after an incubation time of more than 90 days (fig. 1) under aerated, humid conditions. During the first three

*Table 1: Mean pH values of the tested substrates*
*Tab. 1: pH-Mittelwerte der untersuchten Substrate*

| Substrate | pH value |
|---|---|
| Sandy soil | 7.6 |
| Sand substrate | 7.7 |
| CaO substrate | 12.6 |

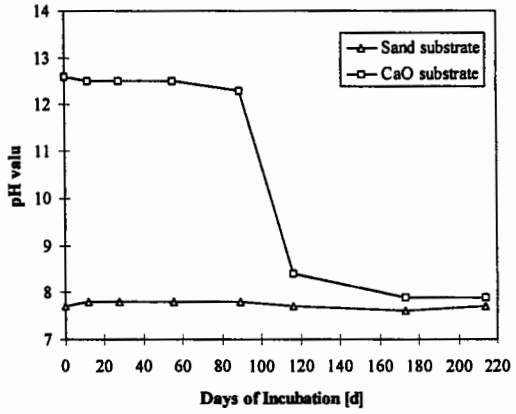


*Fig. 1: pH value in the sand substrate and the CaO substrate*
*Abb. 1: pH-Werte des Sand-Substrates und des CaO-Substrates*

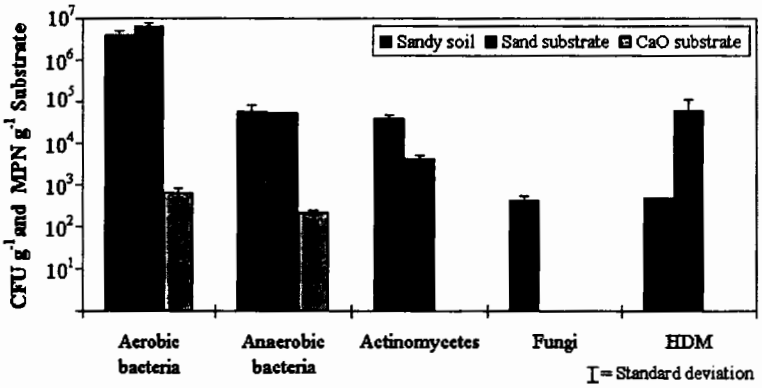


*Fig. 2: Number of microbes in an unpolluted soil (sandy soil), a diesel fuel polluted soil (sand substrate) and a diesel fuel polluted soil amended with hydrophobised CaO (CaO substrate)*
*Abb.2: Mikrobielle Keimzahlen des unkontaminierten Bodens (Sandboden), des mit Diesel-Kraftstoff kontaminierten Bodens (Sand-Substrat) und des mit Diesel-Kraftstoff kontaminierten Bodens bei Zusatz von hydrophobiertem CaO (CaO-Substrat)*

months the pH decreased only slightly from 12.6 to 12.3. When the same substrate was incubated under anaerobic conditions, no decrease of the pH value was observed even after more than 3 years of experimentation. The pH value in the sand substrate stayed stable over 216 days at 7.7 ± 0.1 (fig.1).

*Number of micro-organisms*

The addition of diesel fuel and hydrophobised CaO yielded distinct effects on the colonisation density of different groups of micro-organisms (fig. 2).

The number of heterotrophic aerobic bacteria increased slightly from $4 \cdot 10^6$ up to $6.4 \cdot 10^6$ $g^{-1}$ CFU after the addition of diesel fuel, but the number of anaerobic bacteria decreased slightly by about 6 % from $5.7 \cdot 10^4$ to $5.3 \cdot 10^4$ $g^{-1}$ CFU. The addition of hydrophobised CaO to the polluted soil decreased the number of heterotrophic aerobic and anaerobic bacteria by approximately 99 %. Only $9.9 \cdot 10^2$ $g^{-1}$ CFU of aerobic bacteria and $3.1 \cdot 10^2$ $g^{-1}$ CFU of anaerobic bacteria were counted (fig. 2).

The addition of diesel fuel suppressed the colonisation with actinomycetes in the investigated sand substrate from $3.9 \cdot 10^4$ to $4.4 \cdot 10^3$ $g^{-1}$ CFU. The population of fungi was completely destroyed. After treatment of the polluted soil with hydrophobised CaO neither fungi, nor actinomycetes were found (fig. 2).

The addition of diesel fuel increased the number of hydrocarbon degrading micro-organisms as expected. MPN increased from $4.8 \cdot 10^2$ up to $6.3 \cdot 10^4$ $g^{-1}$. When hydrophobised CaO was added, no HDM were detected (fig. 2).

Figure 3 shows the number of micro-organisms in the sand substrate (3a) and the CaO substrate (3b) after long-

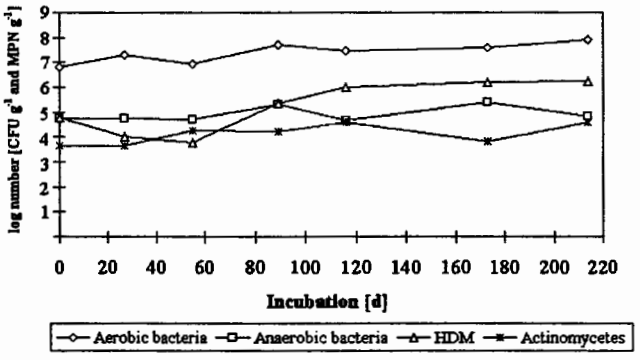


*Fig. 3a: Number of microbes of different groups in the sand substrate*
*Abb. 3a: Mikrobielle Keimzahlen unterschiedlicher Gruppen im Sand-Substrat*

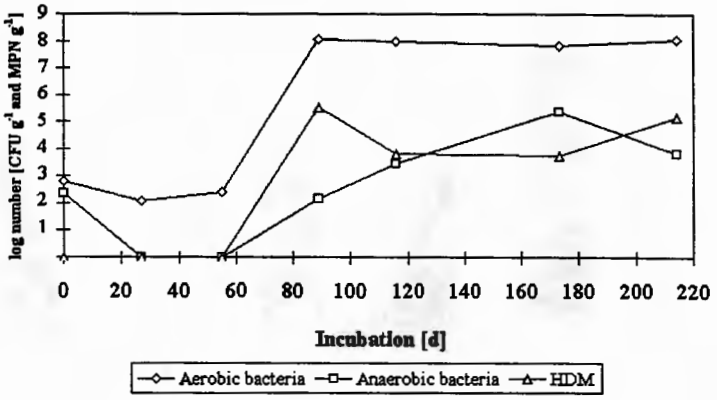


*Fig. 3b: Number of microbes of different groups in the CaO substrate*
*Abb. 3b: Mikrobielle Keimzahlen unterschiedlicher Gruppen im CaO-Substrat*

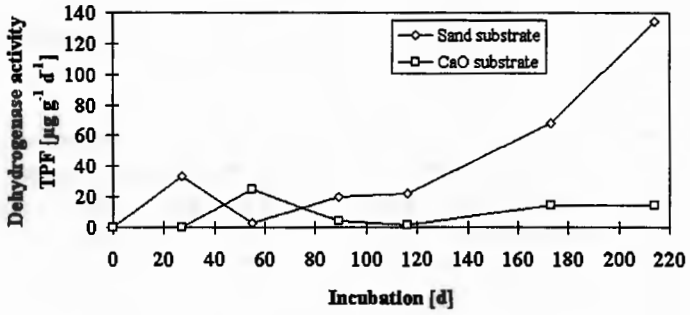


*Fig. 4: Dehydrogenase activity in the sand substrate and the CaO substrate in relation to incubation time*
*Abb.4: Dehydrogenase-Aktivität des Sand-Substrates und des CaO-Substrates während der Inkubation*

term incubation. The number of different micro-organisms varied only slightly in the sand substrate. The number of aerobic bacteria was stable at about $10^7$ up to $10^8$ $g^{-1}$ CFU. The number of anaerobic bacteria amounted to about $10^5$ $g^{-1}$ CFU. About $4.4 \cdot 10^4$ up to $3.9 \cdot 10^4$ $g^{-1}$ CFU were counted in case of actinomycetes.

Only the MPN of hydrocarbon degrading micro-organisms (HDM) decreased from $3 \cdot 10^4$ to $5.9 \cdot 10^3$ $g^{-1}$ within the first 60 days of incubation. From then onwards the MPN of HDM increased up to $1.7 \cdot 10^6$ $g^{-1}$ until the end of the incubation. Fungi seemed to be sensitive to diesel fuel as none was found in the sand substrate, while the sandy soil contained fungi before pollution (fig. 2).

The number of microbes in the CaO substrate was clearly lower than that of the sand substrate at the start of the incubation. The number of aerobic bacteria increased from $6.5 \cdot 10^2$ $g^{-1}$ CFU to about $10^8$ $g^{-1}$ CFU after 60 days of incubation in the CaO substrate. This value was higher than that found in the sand substrate (fig. 3a). Anaerobic bacteria decreased from $2.2 \cdot 10^2$ $g^{-1}$ CFU to zero after 25 days but were found again after 60 days of incubation and reached a maximum value of $2.3.10^5$ $g^{-1}$ CFU. Hydrocarbon degrading micro-organisms were found first after 90 days of incubation. The highest MPN was $3.5 \cdot 10^5$ $g^{-1}$ and after the incubation period a value of $1.5 \cdot 10^5$ $g^{-1}$ was deter-

*Table 2: Metabolic dehydrogenase activity and oxygen demand before and after pollution of a sandy soil with diesel fuel and subsequent CaO-treatment*
*Tab.2: Dehydrogenase-Aktivität und Sauerstoffbedarf vor und nach der Kontamination eines Sandbodens und anschließender CaO-Behandlung*

| | Dehydrogenase activity [µg $g^{-1}$ $d^{-1}$ TPF*] | Oxygen demand [mg $kg^{-1}$ $h^{-1}$ $O_2$] |
|---|---|---|
| Sandy soil | 11.3 | 0.03 |
| Sand substrate | 0 | 3.8 |
| CaO substrate | 0 | 0 |

*TPF = Triphenylformazan

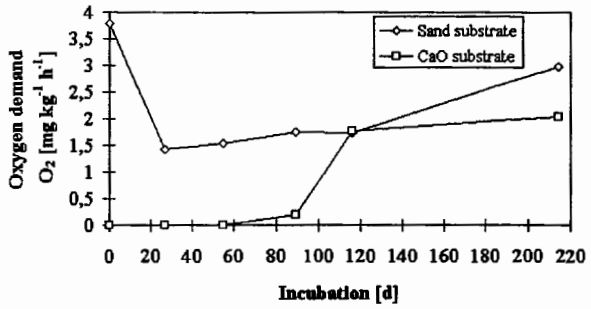


*Fig. 5: Oxygen demand of the sand substrate and the CaO substrate in relation to incubation time*
*Abb.5: Sauerstoffbedarf des Sand-Substrates und des CaO-Substrates während der Inkubation*

mined. Neither fungi, nor actinomycetes were in the CaO substrate (fig. 3b).

*Microbial activity*

The microbial activity of micro-organisms was determined indirectly by measuring the dehydrogenase activity and the oxygen demand.

The dehydrogenase activity and the oxygen demand of the unpolluted sandy soil were very low. This might be due to the extreme low carbon content.

The addition of diesel fuel deactivated the dehydrogenase, while the oxygen demand increased more than 100-fold (tab. 2). This result indicates that diesel fuel was used as a carbon source by the micro-organisms.

The addition of hydrophobised CaO decreased the enzymatic activity and the oxygen demand to a level beyond the detection limit (tab. 2).

No dehydrogenase activity was measured in the sand substrate and in the CaO substrate at the start of the incubation (fig. 4). The dehydrogenase activity increased from zero to 33.1 µg $g^{-1}$ $d^{-1}$ TPF after 25 days of incubation in the sand substrate. Afterwards the activity decreased until 55 days after start of the experiment before it continuously increased again. The maximum dehydrogenase activity was 135 µg $g^{-1}$ $d^{-1}$ TPF.

The dehydrogenase activity was lower in the CaO substrate and was above the detection limit first after 55 days of incubation with a value of 25 µg $g^{-1}$ $d^{-1}$ TPF. After 220 days 14 µg $g^{-1}$ $d^{-1}$ TPF were determined (fig. 4).

The oxygen demand decreased from 3.8 g $kg^{-1}$ $h^{-1}$ to 1.4 mg $kg^{-1}$ $h^{-1}$ $O_2$ in the sand substrate within the first four weeks after incubation (fig. 5). Afterwards the oxygen demand continuously increased up to 3 mg $kg^{-1}$ $h^{-1}$ $O_2$.

After 60 days of incubation an oxygen demand was determined in the CaO substrate. After 118 days of incubation, the oxygen demand showed a similar as that in the sand substrate (1.7 mg $kg^{-1}$ $h^{-1}$ $O_2$) which is probably related to changes in soil pH (fig. 1). The highest oxygen demand was determined at the end of the incubation phase with 2 mg $kg^{-1}$ $h^{-1}$ $O_2$ (fig. 5).

*Table 3: Correlation coefficients of pH, number of microbes, oxygen demand and dehydrogenase activity in the CaO substrate*
*Tab. 3: Korrelationskoeffizienten von pH, Keimzahlen, Sauerstoffbedarf und Dehydrogenaseaktivität des CaO-Substrat*

| | pH | Aerobic bacteria | Anaerobic bacteria | HDM | Oxygen demand | Dehydrogenase activity |
|---|---|---|---|---|---|---|
| pH | - | -0.62 | -0.52 | 0.09 | -0.99*** | -0.19 |
| Aerobic Bacteria | | - | 0.11 | 0.69 | 0.70 | -0.02 |
| Anaerobic Bacteria | | | - | -0.22 | 0.92* | -0.29 |
| HDM | | | | - | 0.07 | -0.07 |
| Oxygen demand | | | | | - | -0.05 |

Note: * and *** significant at p ≤ 5 % and ≤ 1 % respectively

## Discussion

The addition of 2 % diesel fuel had no effect on the soil pH value, but increased strongly after the addition of hydrophobic CaO to sand substrate (tab. 1). The subsequent decrease of the pH value after the long-term incubation of the soil material is a chemical process caused by an exothermic reaction of CaO, soil water and $CO_2$ (fig. 1). First, CaO reacts $H_2O$ to $Ca(OH)_2$. Afterwards $Ca(OH)_2$ and $CO_2$ create $CaCO_3$ and $H_2O$. The $CO_2$ could derives from the air or was produced by aerobic metabolism of micro-organisms. The lacking oxygen demand until day 90 of incubation, however, verified that the $CO_2$ must derives from the air only.

The hydrophobic character of CaO impedes an immediate reaction with soil water. This will start after degradation of the stearic acid which starts exclusively by chemical hydrolysis as no microbial activity was observed at the start of the experiment. The stearic acid was completely degraded after 90 days (fig. 1).

CaO damaged the microbial community to a higher extend than did pollution with 2 % diesel fuel (fig. 2). Diesel fuel even had a promoting effect on the number of aerobic bacteria and HDM (hydrocarbon degrading micro-organisms). This increasing effect of hydrocarbons is well known (Bossert and Bartha, 1984; Pfaender and Buckley, 1984). The number of anaerobic bacteria decreased slightly, that of actinomycetes more pronounced after addition of diesel fuel. The community of fungi was destroyed completely by diesel fuel.

The treatment of the sand substrate with hydrophobised CaO increased the soil pH drastically and thus influenced strongly the growth conditions of the microbes. The number of aerobic and anaerobic bacteria decreased by about 99 % (fig. 2); the population of HDM was completely

destroyed. But the population of these microbes regenerated as the long-term incubation experiment showed. The population of fungi and actinomycetes was destroyed, however, irreversibly.

Bacteria and actinomycetes prefer soil pH values between 6 and 9, fungi pH values of ≤5.5 (Metting, 1993). Alexander (1977) described fungi as intolerant with view to alkaline growth media. Costa et al. (1988) doubted the growth of micro-organisms in environments with pH values ≥11.5. Obviously the bacteria found after the treatment with CaO survived in form of spores and in micro-habitats, respectively. For the variability of pH values within such micro-habitats Lynch (1988) gave a range of 2 pH units.

With the start of the decrease of the pH value in the incubation experiment, the number of aerobic and anaerobic bacteria and HDM increased again (fig. 3b). A negative but not significant correlation was found between pH of the CaO substrate and number of aerobic bacteria and anaerobic bacteria, respectively (tab. 3).

The number of micro-organisms provides no information about their metabolic activity. Gray and Williams (1971a) stress that micro-organisms which grow on plates may be in dormancy under natural environmental conditions. Therefore Alexander (1977) suggested to measure besides the number of microbes, the enzyme activity and respiration rate.

Activity of dehydrogenase is thought to reflect total oxidative activity of soil micro-organisms (Ladd, 1978). In accordance to findings of Weissmann et al. (1994) dehydrogenase activity of the sand substrate increased after application of hydrocarbons. Weissmann et al. (1994) put this increase down to degradation of hydrocarbons by micro-organisms.

The dehydrogenase activity in the CaO substrate increased only slightly within time and remained on a low level. No significant correlations of dehydrogenase activity and pH, microbial number or oxygen demand were determined (tab. 3) which is in line with the results of Pitchel and Hayes (1990). Changes of the dehydrogenase activity in the sand and the CaO substrate may be due to the easily degradable fraction of the added diesel fuel.

The measurement of the oxygen demand or $CO_2$ production is the most reliable method to evaluate the metabolic activity of micro-organisms (Gray and Williams, 1971b). An increasing oxygen demand after the addition of diesel fuel was noticed in other experiments, too (e.g. Miethe et al., 1994; Stegmann et al., 1991; Hupe et al., 1996). This result indicates that diesel fuel was degraded by micro-organisms which used the diesel fuel as a carbon or energy source. The oxygen demand of the CaO substrate was low, because the amendment of CaO damaged the micro-organisms. A close and negative correlation existed between pH and oxygen demand ($r = -0.99^{***}$; tab. 3). The number of anaerobic bacteria was positively correlated to the oxygen demand ($r = 0.70$). A significant relationship ($r = 0.92^{*}$) was found for oxygen demand and number of anaerobic bacteria which might have been caused by facultative anaerobic bacteria.

## Conclusions

Overliming with hydrophobised caustic lime which was applied in order to immobilise diesel fuel damaged micro-organisms effectively as the determination of the number of different microbes, the dehydrogenase activity and the oxygen demand revealed. The addition of hydrophobised CaO damaged micro-organisms to a greater extent than did the pollution with 2 % diesel fuel. Therefore the general recommendation of overliming contaminated soils needs to be evaluated critically, especially as these soils still contain the pollutant. A release of the diesel fuel by weathering may even promote adverse effects as the number and activity of the micro-organisms was reduced by hydrophobised CaO before. The fate of such CaO substrates used in road construction is unknown, but such wastes should not enter the biocycle again.

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