Università degli Studi di Napoli "Federico II"
Facoltà di Ingegneria

Katholieke Universiteit Leuven
Faculty of Bioscience Engineering

CORSO DI LAUREA IN INGEGNERIA PER L'AMBIENTE E IL TERRITORIO

DIPARTIMENTO DI INGEGNERIA IDRAULICA, GEOTECNICA ED AMBIENTALE

Tesi di Laurea Specialistica

Survival and activity of phenanthrene degrading *Sphingomonas* sp. LH128 in soil microcosms

Abstract

Relatori:
Prof. Dr. Ing. Massimiliano Fabbricino
Prof. Dr. Ing. Dirk Springael

Candidato:
DENIS TRANI
matricola 324/183

Correlatore:
Dr. Ing. Philip Breugelmans

Anno Accademico 2009 - 2010

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread pollutants that are potentially hazardous to human health. Due to their toxic, mutagenic, and carcinogenic properties, there is much interest in understanding their biodegradation mechanisms and their environmental fate. For the same reasons, remediation of contaminated sites is important.

The term PAH covers different classes of organic compounds and PAHs are generally described as molecules which consist of two or more fused aromatic rings in various structural configurations. They contain by definition only carbon and hydrogen atoms. Polycyclic aromatic hydrocarbons are naturally occurring compounds produced continuously due to inadvertently incomplete combustion of organic matter and are therefore very common in the environment. Besides natural emission sources such as forests fires and volcanoes, anthropogenic activities have contributed to the contamination.

PAHs can be divided into 2 major groups: Low Molecular Weight (LMW) and High Molecular Weight (HMW) PAHs. The former are characterized by 2 or 3 fused rings, while the latter consist of 4 or more fused rings. PAHs are generally poorly soluble in water and have a high solid-water distribution ratio, which favors their accumulation in the solid phase of the terrestrial environment (on particulates and on humic matter or solubilized in any oily matter which may contaminate water, sediments and soil), making them less susceptible to chemical degradation, and especially biological degradation since microorganisms are prevented from access to PAHs and degrade them. The solubility of PAHs in water is inversely proportional to the number of rings they contain. The aqueous solubility and, as a consequence, the bioavailability of the PAHs decreases almost logarithmically with increasing molecular mass. As a result, HMW PAHs ($\geq$ 4 rings) are almost exclusively bound to particulate matter, while LMW PAHs (up to 3 rings) can also be found dissolved in water. Accordingly, LMW PAHs are sensitive to biodegradation while HMW PAHs seem to be more recalcitrant to microbial degradation. In addition, prolonged aging time in contaminated soils promotes the sequestration of PAH molecules into micropores and increases the recalcitrance of PAHs towards treatment due to the lowered bioavailability. Due to PAH deposition and re-volatilisation in the atmosphere, PAHs can be found in various compartments of the environment: air, surface water, sediment, soil, and consequently also in food and in lipid tissues of both aquatic and terrestrial organisms.

The need to develop practical bioremediation strategies especially for heavily impacted sites is evident. Indeed, some PAHs are known to exert acutely toxic effects and/or possess mutagenic, teratogenic, or carcinogenic properties. Fortunately, PAH degradation potential by micro-organisms is present in the terrestrial environment which ensures natural PAH dissipation. Attention has been mostly paid to the aerobic metabolism. At present, many microorganisms are known to have the enzymatic capacity to oxidize PAHs that range in size from naphthalene (2-ring) to benzo[a]pyrene (5-ring). Given the requisite environmental conditions, microbial communities are able to readily degrade these chemicals. Bacteria that are able to use PAHs as the sole source of carbon and energy were frequently isolated from PAH-contaminated soils. Members of the genera *Pseudomonas*, *Sphingomonas* and *Mycobacterium* are best represented. These

bacteria are considered to be important PAH-degrading colonizers of PAH-contaminated soil which indicates that they are adapted to the low bioavailability of PAHs. A variety of anthropogenically contaminated environments have been reported to harbour *Sphingomonas* strains. Nonetheless, Sphingomonads are also widespread in unpolluted environments such as agricultural soil, water distribution systems and marine environments. Therefore, it seems that members of the genus *Sphingomonas* are adapted to oligotrophic environments. Given their unique properties, PAH-degrading *Sphingomonas* strains might be applied in bioremediation technologies.

By definition, bioremediation involves the use of living organisms, especially microorganisms, to degrade or transform organic environmental contaminants into less harmful/non-hazardous substances which can then be integrated into natural biogeochemical cycles. The use of microorganisms for the bioremediation of soils contaminated with toxic pollutants might in some cases be a good alternative to conventional techniques such as incineration for the recovery or reclamation of land since it can be done at a considerable lower cost and can be regarded as an environmental friendly method. The ability of soil microorganisms to degrade a wide variety of toxic pollutants has been well documented over the past decades. However, biodegradation of complex organic compounds in for example soil is not an intrinsic property and can not be predicted without first delineating the environment in which the contamination is located. Besides the lack of a suitable environment, specialized microbes might be required to degrade certain molecules to harmless compounds.

Many studies have been conducted to establish the effect of bioaugmentation, i.e. inoculation of a contaminated environment with specialized bacteria, on pollutant removal, yet the results of these studies are inconsistent. The reasons for the success or failure of bioaugmentation experiments are not clear. Most successful studies were conducted under controlled laboratory conditions generally favourable to microbial growth and metabolism, yet from these studies the effectiveness of the same strains under field conditions can not be guaranteed. On the other hand, in cases where the inoculation of the soil did not have a positive outcome, the specific reasons for the failure of the inoculum to survive or proliferate generally were not identified.

Objective of this Study

As with all new technologies, successful development requires that the problems and limitations associated with the technique, in this case bacterial inoculation into soil and their survival and activity, are clearly identified to determine whether viable solutions or alternatives can be found. From literature, it is clear that both the biotic and abiotic properties of a particular soil determine the survival and the (degradation) activity of (introduced) bacteria. It has been shown that bacterial degradation activity is affected significantly by the physical and chemical environment which is in soil highly heterogeneous. Yet, the magnitude of these parameters has not been quantified or classified, and the study of these factors can be of crucial importance for improving the survival and proliferation of any particular inoculant strain and to improve engineering strategies to increase the rate and extent of microbial soil remediation.

The purpose of this research was to investigate the influence of different physico-chemical soil parameters on the survival and degradation activity of the phenanthrene degrading soil isolate *Sphingomonas* sp. LH128, and to identify the most important of these factors through multivariate statistical analysis. In addition, the effect of different soil water contents on the survival and activity of the *Sphingomonas* strain was evaluated.

Experimental Setup

The phenanthrene degrading strain *Sphingomonas* sp. LH128 was inoculated at a concentration of $10^7 – 10^8$ colony forming units (CFU) g^{-1} dry soil in sterile soil microcosms (freshly spiked with 500-600 mg phenanthrene kg^{-1} dry soil). The microcosm consisted of a Pyrex tube containing 1 g soil. Two microcosm experiments were conducted, i.e. one to determine the effect of different water levels on the survival and activity of strain LH128 in 3 different soils, and another experiment with 20 soils at a specific moisture content to identify the principal soil parameters influencing strain LH128's survival and activity. At different time points, three tubes per soil and treatment were sacrificed to determine viable (cultivable) cell numbers by plating and residual phenanthrene concentrations using high performance liquid chromatography (HPLC).

Results and Discussion

To determine the influence of the moisture content (MC) on survival and activity of *Sphingomonas* sp. LH128, three autoclaved soils (i.e., soil 151, 152 and 283) were wetted to different extents, relative to their water holding capacity (WHC) at pF 2. Used moisture contents were 0% water, 25% WHC, 50% WHC, 100% WHC and 200% WHC. Soil microcosms were incubated statically at 20 °C for 35 days during which 7 measurements (plating and HPLC at day 0.125, 2, 5, 9, 15, 22 and 35) were performed. Cultivable cell numbers and phenanthrene degradation activity of *Sphingomonas* sp. LH128 in soils 151, 152 and 283 during the incubation period are shown in Fig.1 for each of the 5 moisture contents.

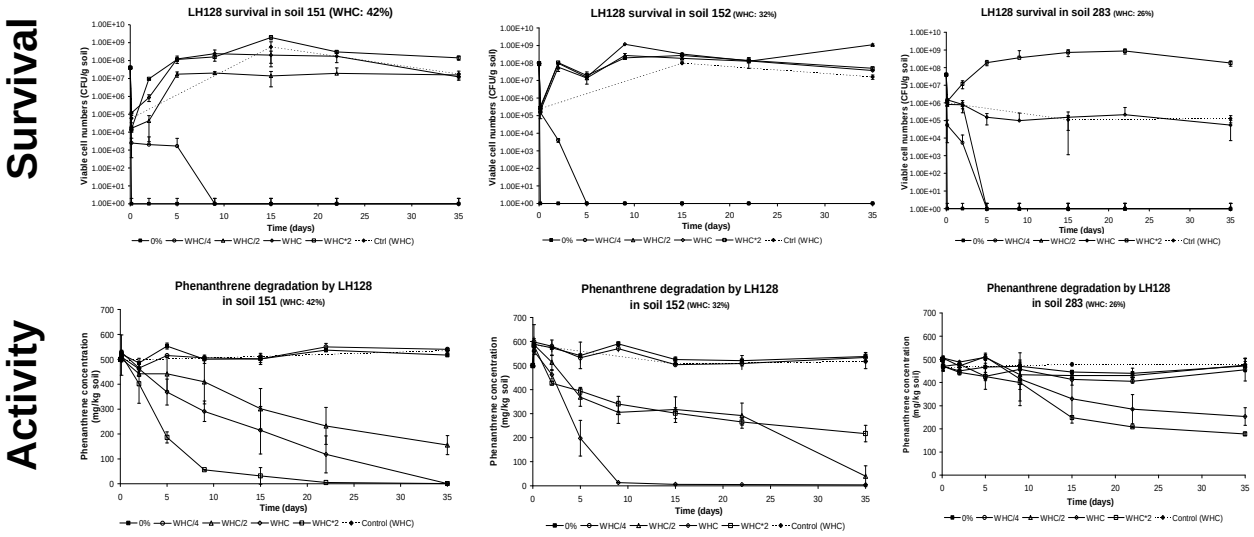

Fig.1 Cultivable cell numbers (CFU/g soil) of *Sphingomonas* sp. LH128 and Phenanthrene concentration (mg/kg soil) over time in soils 151, 152 and 283 for each moisture content at different sampling times (i.e., at day 0.125 (3 hours), 2, 5, 9, 15, 22 & 35).

In all three soils, it was observed that in case no moisture was present (or more precisely, 2% water resulting from the inoculation), *Sphingomonas* sp. LH128 did not survive the inoculation procedure. For the other moisture contents, CFU numbers dropped several orders of magnitude below the inoculum size at the first sampling point, i.e. three hours after inoculation. In soil 283, the inoculum survival rate was the highest. However, the higher inoculum survival rates in soil 283 compared to soils 151 and 152 did not ensure the highest phenanthrene degradation efficiency (see further). In the second driest condition, i.e. 25% WHC (which corresponded to 11%, 8% and 6 % moisture for soil 151, 152 and 283 respectively), strain LH128 persisted only for a few days after which cell numbers fell below the detection level. In these cases, obviously no phenanthrene was degraded. In the 50% WHC condition of soil 283 (13% MC), similar observations were made. It is clear that establishment of the inoculum requires a minimal amount of water. From the experiments performed in this study, it seems that an absolute moisture content of approximately 15% is minimally required to sustain growth over a longer period of time for *Sphingomonas* sp. LH128 in soil.

In the remaining conditions, i.e., the three wettest conditions for soils 151 and 152, and the two wettest conditions in soil 283, cell numbers increased quickly after the initial decline after inoculation. At 100% WHC and 200% WHC for all three soils and also at 50% WHC for soils 151 and 152 LH128 cell numbers increased to 10^6 - 10^9 CFU g^{-1} dry soil and these cell numbers were maintained throughout the experiment. Overall, there was no clear relationship between total cell numbers and moisture content. In addition, the extent and rate of degradation seemed not to be related to the abundance of LH128 cells or moisture content. Indeed, high cell counts did not necessarily entail a high phenanthrene degradation activity. As such, the differences observed in degradation rate and extent result from a different specific phenanthrene degradation activity (i.e., the degradation activity per cell). Only for soil 283 a correlation with cell numbers, moisture content and overall degradation activity was noted.. In this soil, most phenanthrene was degraded in the 200% WHC (51% MC) condition and less at 100% WHC (26% MC). However, the specific degradation activity at 100% WHC was calculated to be higher than at 200 % WHC. The highest observed degradation extent in soil 283 was only 63% of the added amount of phenanthrene after 35 days in the 200% WHC condition (51% MC), and only 47% in the 100% WHC condition (26% MC)..On the other hand, in soils 151 and 152 nearly complete phenanthrene degradation was achieved under some conditions. In addition, the maximal degradation rates achieved in soil 283 did not reach up to those observed in soils 151 or 152. The lower degradation efficiency in soil 283 could not be explained by differences in absolute moisture content since similar degradation rates and even higher degradation extents were observed in soil 151 and especially soil 152 at lower absolute moisture contents. The best phenanthrene degradation was observed at the 100% WHC condition (32% MC) in soil 152, displaying the highest maximal degradation rate observed over all soils and moisture conditions (i.e., 62 mg phenanthrene per day) with nearly complete phenanthrene degradation within 9 days. While for soils 151 and 283 the activity increased with the % of water, for soil 152 this was not observed, probably because in the saturated condition (200% WHC; 63% MC) oxygen could not diffuse easily through the soil profile.

In contrast to most other studies, the inherent capacity of the soil to hold water was taken into account and it was expected that for a given soil the highest cell counts and activity would be observed at the moisture level corresponding to the soil's WHC at pF 2 (corresponding to the moisture content of the soil at field capacity). Indeed, a moisture content corresponding to 40% to 60% of the WHC at pF 1 (which corresponds approximately to the WHC at pF 2) is considered to result in the highest (micro)biological activity. Based on the results obtained in the present study, it cannot be concluded that the highest microbiological activity is achieved at a moisture level corresponding to 100% WHC at pF 2 (~FC) , nor at a moisture level corresponding to 50% WHC at pF 2 (~50% FC) as reported previously. Overall, it can be concluded in agreement with other studies that the amount of available water affects the biodegradation activity; either at too low levels the water activity is inadequate resulting in desiccation stress, or at too high levels insufficient oxygen can be supplied which is required for aerobic degradation processes. However, the optimal water content cannot be easily predicted. Indeed, soil is a complex environment and it is important not to neglect its inherent heterogeneity and take into account that the addition of the same absolute water content to physico-chemical diverse soils will have a different effect on the soil's properties (for example, water activity, diffusion processes,…). The physico-chemical soil characteristics, which are directly or indirectly related to the available amount of water, can have an important effect on the survival and activity on the soil microbial community and must be considered when evaluating the performance of introduced bacteria. In this perspective, a microcosm experiment with 20 soils with diverse physical and chemical properties was performed.

To determine the influence of physico-chemical soil characteristics on survival and activity of *Sphingomonas* sp. LH128 twenty γ-irradiated soils with varying physico-chemical properties were wetted to 100% of their WHC (pF 2). Soil microcosms were incubated statically at 25 °C for 35 days. During this period viable cells were enumerated and phenanthrene concentrations were determined at three time points, i.e. at 1 day, 10 days and 20 days after inoculation by means of plating and HPLC analysis, respectively. The survival of *Sphingomonas* sp. LH128 and the phenanthrene removal pattern in the 20 soils at the different time points are shown in Fig.2.

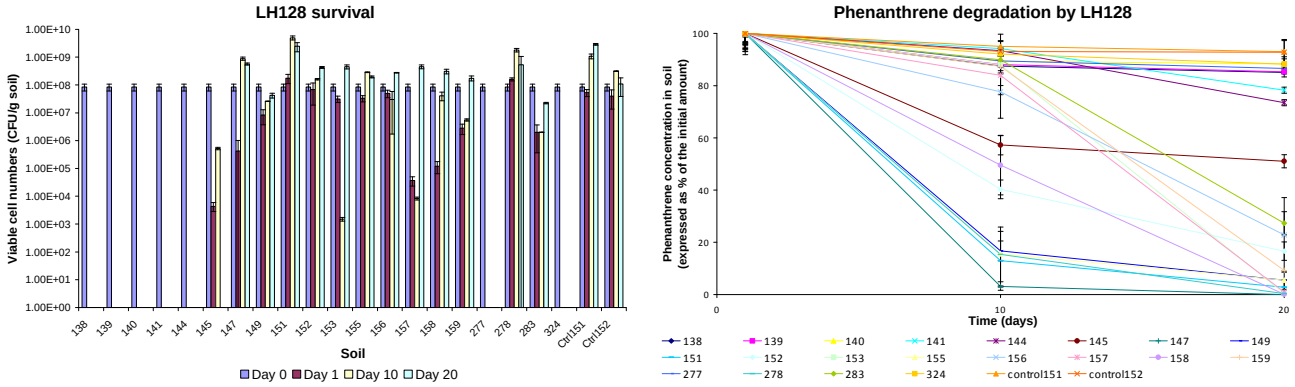

Fig.2 Survival of *Sphingomonas* sp. LH128 and phenanthrene removal pattern in the 20 soils at the different time points (i.e., at day 1, 10 & 20).

Considerable variation in LH128 inoculum survival, growth and activity was observed between the different soils. The type of soil had a large influence on the initial survival rates of introduced LH128 cells and determined the persistence and growth of the *Sphingomonas* strain. Clearly, soil pH is a crucial factor controlling inoculum survival and growth. A pH lower than 5 impaired growth of *Sphingomonas* sp. LH128. However, pH alone could not explain the observed variability in the other soil samples with higher pH values so other soil parameters must be at play here.

In the 20 soils, phenanthrene was degraded to different extents and at different rates. Several degradation patterns could be distinguished. At day 10, three groups could be identified: (1) soils in which no or limited degradation occurred, (2) soils in which degradation was mediocre, and (3) soils in which high degradation activity was present. When considering the evolution in phenanthrene concentration in the final 10 days, some groups could be further subdivided. Indeed, in some of the soils where no degradation was observed at day 10, phenanthrene degradation was initiated eventually, even at considerable high rates. In the soils showing mediocre degradation at day 10, continued activity was observed in some soils while in others degradation was halted. As such, in general, 5 groups could be identified.

Based on the comparison between cell numbers and degradation activity, it is clear that in some soils there was a correspondence between phenanthrene degradation and bacterial growth, but this relationship was not always true. In some soils no phenanthrene degradation could easily be linked to the absence of detectable numbers of *Sphingomonas* sp. LH128 cells at the different measuring points, while in other soils a very limited degradation activity was observed although high cell numbers were detected, suggesting that in some way the physico-chemical composition of the soil inhibited phenanthrene degradation by strain LH128. As such, even when many cells with specific catabolic properties are present good phenanthrene degradation is not guaranteed.

The different degradation patterns and activity observed in the different soils and the lack of correlation between bacterial activity and survival are a result of the diverse physico-chemical conditions in the soils. However, it is difficult to determine by visual inspection of the soil parameters which factors determine death or survival and activity of the introduced *Sphingomonas* strain. For this reason, multivariate regression analysis was be performed against an extensive set of soil parameters to objectively determine the principal factors influencing survival and degradation activity of strain LH128 in phenanthrene-spiked sterile soil microcosms.

Multivariate statistical analysis using Principal Component Regression (PCR) analysis and Partial Least Square Regression (PLSR) analysis allowed the identification of the most important abiotic soil parameters. PLSR resulted in a more accurate predictive model than PCR for both cultivable cell numbers and phenanthrene degradation activity. Among others, pH, exchangeable Al concentrations, oxalate extractable Mn concentrations, organic carbon content, nitrogen content and also the soil particle size distribution were the most important soil characteristics.

General Conclusions and Future Perspectives

It was shown in this study that a minimal moisture content is required to allow establishment and growth of introduced bacteria in sterile soil microcosms. However, it was not possible to uniformly identify the optimal moisture content which will ensure most efficient pollutant degradation. Overall, a moisture content corresponding to 100% – 200% of the soil's water holding capacity (at pF 2) can be considered as a suitable moisture content. However, other abiotic soil characteristics must be considered among which soil pH was shown to be of critical importance.

Through multivariate statistical analysis the most important factors in an extensive set of physical and chemical soil characteristics influencing the survival and growth, and phenanthrene degradation activity of *Sphingomonas* sp. LH128 could be identified and accurate predictive models based on PLSR could be constructed. As such, this work contributes to the understanding how bacteria - artificially introduced into the environment - will respond in terms of growth and activity to the prevailing soil conditions, and this knowledge could be applied to assess the feasibility of bioaugmentation at contaminated sites taken into account the prevailing environmental conditions at a contaminated site. In addition, it was noted in this study that high numbers of bacteria with specific catabolic properties does not necessarily entail high degradation efficiency. Indeed, several factors could inhibit pollutant degradation such as catabolic repression or lack of sufficient and/or specific nutrients. This is of importance since if the presence and/or abundance of pollutant-degrading bacteria present at contaminated sites is used as an indicator for degradation potential, the bioremediation efficiency might be overestimated.

In the future, more soil parameters (such as total phosphorus content, available organic phosphorus and soil pore water composition) will be analyzed in order to further optimize the predictive model. In addition, the influence of several biotic soil parameters, such as total cell numbers and bacterial diversity, on the survival and activity of *Sphingomonas* sp. LH128 will be analyzed. Indeed, both the abiotic and the biotic conditions in the soil will determine the performance of introduced bacteria with catabolic properties of interest and under real life conditions all factors need to be taken into account.