Groundwater bacteria

Diversity, activity and physiology of pesticide degradation at low concentrations

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DTU Environment
Department of Environmental Engineering

PhD Thesis
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Preface

This thesis comprises the research done for a PhD project undertaken from 2009 to 2012 at DTU Environment, Technical University of Denmark. The thesis was funded by the GOODWATER project of European Commission's Marie Curie Initial Training Network for young scientists under grant no. 212683. The experiments presented herein were performed at the Department of Geochemistry, Geological Survey of Denmark and Greenland (GEUS). The study included an external research visit at Ghent University, Faculty of Bioscience Engineering, The Laboratory of Microbial Ecology and Technology (LabMET) between April and July 2011. The primary supervisor was Professor Hans-Jørgen Albrechtsen, and the co-supervisors were Dr. Sebastian R. Sørensen and Professor Jens Aamand from GEUS. The supervisor during the external research visit was Professor Nico Boon.

This thesis is based on 3 journal Papers (Paper I, II and III).

I. Gözdereliler, E., Boon, N., Aamand, J., De Roy, K., Granitsiotis, MS., Albrechtsen, HJ., Sørensen, SR. Comparing metabolic functionality, community structure and dynamics of herbicide-degrading communities cultivated with different substrate concentrations, submitted manuscript.

II. Gözdereliler, E., Dechesne A., Nicolaisen, MH., Sharp, RE., Aamand, J., Albrechtsen, HJ., Smets, B., Sørensen, SR. Isolation of 2-methyl-4-chlorophenoxyacetic acid degrading bacteria from groundwater sediments using a novel low substrate flux approach, submitted manuscript.

III. Gözdereliler E, De Roy K, Boon N, Sørensen SR. Substrate concentration alter the cytometric characteristics of herbicide degrading bacteria with high and low nucleic acid content, submitted manuscript.

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment. Contact library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2000 Kgs. Lyngby, Denmark.

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My deepest thanks to my parents and all my family for their unconditional support and love. I want to thank you Suzi – This thesis could not have been written without the understanding, patience and love of yours.

Finally, I dedicate my work to my dear uncle, Tarık Gözdereliler, may he rest in peace.

Christianshavn, June 2012

Erkin Gözdereliler

When I say harbour
Why do I think of masts
And of sails when I say high seas?

And of cats when I say March
And of rights when I say workers?
Why does the old miller
Believe in God without a thought?

And on windy days
Why does rain come down at slant?

Orhan Veli Kanık

Abstract

Pesticide contamination of groundwater is a well-documented and extensive pollution problem characterized by low chemical concentrations. As a consequence of widespread and repeated use, pesticide residues are frequently detected in surface- and groundwaters serving as public drinking water resources, in the concentration range of nano- to micrograms per liter. Such concentrations may still exceed the European drinking water limits of 0.1 μg L^{-1} for individual pesticides, and be a threat to drinking water resources.

The behavior, degradation mechanisms and treatment of pollutants occurring at high concentrations (mg L^{-1}) are relatively well understood, however the microbial processes and degradation kinetics of pollutants occurring at low concentrations (pg L^{-1} to μgL^{-1}) can differ significantly from the processes at higher concentrations. Our knowledge on the processes that cause such bottlenecks is very limited and mostly biased by the fact that the concentrations of organic chemicals used in the laboratory are far higher than those found in the environment.

The overall aim of this PhD study was to improve our understanding of degradation of low pollutant concentrations in oligotrophic aquifers, and to understand the mechanisms and bacterial physiology underlying the occurrence of low pollutant concentrations. This may allow us to develop new and more efficient approaches for remediation of pollutants at low concentrations *in situ*.

Due to common use of high substrate concentrations in cultivation studies, bacterial populations existing in the same micro-niches which are adapted to metabolize substrates at low concentrations may have been overlooked and they could be more efficient to remediate low levels of organic pollutants in contaminated environments. In order to demonstrate the effect of substrate concentrations in enrichment processes, we have investigated the differences in metabolic activity, community structure and dynamics, population growth, and single cell physiology of two 4-chloro-2-methylphenoxyacetic acid (MCPA) degrading enrichment cultures. The cultures were obtained by a conventional enrichment approach based on different MCPA concentrations originated from the same aquifer material. We have shown that using low substrate concentrations (100 μg L^{-1}), in contrast to the classical high concentrations (25 mg L^{-1}), provide more efficient and stable bacterial communities in regards metabolic functionality and community structure. Furthermore, we have

demonstrated that low nucleic acid (LNA)-content bacteria proliferated in parallel to mineralization activity in cultures selected on low herbicide concentration, suggesting that LNA bacteria may play a role in degradation of low pollutant concentrations in contaminated sources.

One of the major goals of this thesis was to isolate microbial populations that are adapted to metabolize low pollutant concentrations from oligotrophic environments. In this perspective, we have developed a novel cultivation approach which combines long term cultivation with low diffusive fluxes, in order to demonstrate that groundwater sediments contain an unrecognized diversity of pesticide-degraders. This system is called Low Flux Filter (LFF) plate and it provides a constant flux of MCPA to the bacteria allowing them to grow slowly on a membrane placed on an agar surface, which is transferred to fresh MCPA plates periodically. The isolated strains, the first MCPA-degraders isolated directly on MCPA, were most efficient at mineralizing low MCPA concentrations in line with the nutrient scarcity of their natural habitat.

Finally, we investigated the effect of substrate concentrations on the bimodal distribution of low nucleic acid (LNA) and high nucleic acid (HNA) cells of MCPA degrading bacterial strains in order to get a better understanding of the physiological, ecological and functional relevance of LNA-HNA populations. The results showed that bacterial strains obtained with low MCPA levels were dominated by LNA cells and this dominance decreases with increasing MCPA concentrations. Whereas, the strains obtained with high levels of substrate showed strict HNA characteritics regardless of the concentration. The strains showing LNA characteritics were reported to be more efficient in mineralizing low MCPA concentrations and inhibited by high levels of MCPA. This suggests that bacterial populations harbouring LNA-cells could be possible candidates for bioremediation of environments contaminated with low concentrations of pollutants.

Overall this PhD study showed that aquifers contain microbial populations that are adapted to low pesticide concentrations, whose potential can be accessed using specific cultivation approaches and their characteritics could be identified by the use of state-of-the-art methods. The results of this thesis would contribute significantly to our understanding to develop remediation strategies for degradation of pollutants at low concentrations.

Dansk sammenfatning

Forurening af grundvand med lave koncentrationer af pesticider er et veldokumenteret og udbredt problem. Som en direkte konsekvens af dette, så lukkes flere drikkevandboringer pga. overskridelse af EU grænseværdien på 0,1 $\mu g\ L^{-1}$.

Mens nedbrydningsprocesserne ved høje pesticidkoncentrationer (mg L^{-1}) er velkendte, er nedbrydningen ved lave koncentrationer (pg L^{-1} to $\mu g\ L^{-1}$) mindre kendt. Vores viden om nedbrydningsprocesserne af organiske stoffer ved lave koncentrationer er meget begrænset. I laboratorieforsøg bruges ofte højere koncentrationer end dem fundet naturligt i miljøet, og resultaterne herfra kan derfor være misvisende.

Formålet med denne PhD-afhandling var at forbedre vores forståelse af nedbrydning af forureningsstoffer, der forekommer i lave koncentrationer i de næringsstof-begrænsede grundvandsmagasin, samt at forstå de mekanismer og bakteriernes fysiologi der danner grundlag for forekomst af disse af forureningsstoffer. Dette vil ultimativt muliggøre udvikling af en ny og mere effektive tilgang for *in situ* rensning af de forureningsstoffer med lave koncentrationer.

I traditionelle studier med høje substrat-koncentrationer er der risiko for at små populationer i mikro-nicher overses. Disse populationer vil måske være bedre egnet til at nedbryde substrater ved lave koncentrationer.

For at belyse effekten af substrat-koncentrationen, har vi undersøgt forskelle i metabolisk aktivitet, samfundsstruktur og -dynamik, populationsvækst samt enkel cellefysiologi af to forskellige 4-chloro-2-methylphenoxyacetic acid (MCPA)-nedbrydende kulturer. Kulturerne blev isoleret ved traditionel berigelse med forskellige MCPA-koncentrationer med materiale fra samme akvifer.

Vi har vist, at der ved brug af lave substrat-koncentrationer (100 $\mu g\ L^{-1}$) i stedet for de traditionelt høje koncentrationer (25 mg L^{-1}) opstår et mere effektivt og stabilt bakterielt samfund med hensyn til metabolisk funktionalitet og community struktur. Derudover har vi vist at bakterier med lavt kernesyreindhold (LNA) formerer sig parallelt med mineraliseringsaktivitet i udvalgte kulturer ved lav herbicid-koncentration, hvilket indikerer, at LNA-bakterier måske har en rolle i forbindelse med nedbrydningen af forurenende stoffer ved lav koncentration.

Et af hovedformålene ved denne afhandling var at isolere mikrobielle populationer, som er adapteret til at metabolisere forurenende stoffer ved lave koncentrationer fra oligotrofe miljøer. I denne forbindelse har vi udviklet en opdyrkningsmetode, som kombinerer dyrkning over lang tid med små diffusive fluxe, for at kunne demonstrere at i grundvandssedimenter findes en ikke-belyst diversitet af pesticid-nedbrydere.

Systemet kaldes en Low Flux Filter (LFF) plade, hvor der er en konstant tilførsel af MCPA til bakterierne, hvorved de gror langsomt på membranen placeret oven på agarpladen. Membranen overføres periodisk til friske MCPA plader. De første MCPA-nedbrydere isoleret direkte på MCPA var mest effektive til at nedbryde MCPA ved lave koncentrationer, hvilket stemmer overens med det lave indhold af næringsstoffer fra dens naturlige habitat.

Vi har yderligere undersøgt effekten af substrat-koncentration på en bimodal fordeling af MCPA-nedbrydende bakterier med lavt (LNA) og højt (HNA) kernesyre-inhold for at kunne få en bedre forståelse af fysiologi og funktionel relevans af LNA/HNA populationer. Resultaterne viste, at isolerede bakterier ved lavt MCPA niveau var domineret af LNA celler, og denne dominans faldt med stigende MCPA niveau. Derimod udviste celler isoleret ved høje substrat-koncentrationer HNA karakteristika uafhængig af substratkoncentration. Celler med LNA karakteristika var mere effektive til at nedbryde MCPA ved lave koncentrationer og blev hæmmet ved høje MCPA-koncentrationer. Dette indikerer, at bakterielle populationer med LNA-celler er mulige kandidater til bioremediering af forurenede miljøer med lave koncentrationer.

Overordnet har denne afhandling vist, at akviferer indeholder mikrobielle samfund, som er adapteret til lave pesticid-koncentrationer, hvis potentiale kan undersøges ved at bruge specifikke dyrkningsmetoder, mens deres karakteristika kan identificeres ved hjælp af state-of-the-art metoder. Resultaterne fra denne afhandling bidrager signifikant til vores forståelse af nedbrydning af forurenende stoffer ved lave koncentrationer.

List of contents

1. Introduction

Groundwater is an important drinking water source in many countries not least in Denmark where all the drinking water originates from, and the protection of this resource is a matter of great concern. In Denmark, groundwater undergoes a simple aeration and filtration process before it is distributed to the consumers (Jørgensen and Stockmarr, 2009). This requires high quality groundwater that must meet the quality standards with respect to e.g maximum allowed concentrations of xenobiotics. Pesticides comprise the largest group of xenobiotic compounds deliberately introduced into the environment. Besides the benefits to the increased agricultural activity, the use of pesticides has created potential risks for the environment (Scheidleder et al., 1999; Pazos et al., 2003). The frequent findings of pesticides in Danish groundwater have resulted in the in closure of numerous groundwater abstraction wells or entailing considerable treatment costs. Recent groundwater monitoring program showed that the presence of pesticides has been the major reason for closure of drinking water wells decommissioned due to contamination (Miljøstyrelsen, 2011).

Even though most pesticides are applied in amounts up to several kilos per hectare, the detected residues in groundwater are typically in the nano- to micrograms per liter concentration range (Kolpin et al., 2000; Thorling et al., 2010). Such concentrations may still exceed the European drinking water limits for pesticides and their metabolites (EU 2011) which are 0.1 $\mu g\ L^{-1}$ for each pesticide or metabolite and 0.5 $\mu g\ L^{-1}$ for the sum of pesticides and metabolites, and be a threat to drinking water resources (GEUS 2011; Scheidleder et al., 1999). Figure 1.1 shows the distribution of pesticides in monitoring wells in Denmark between 1993 and 2010. As can be seen from the figure, detection of low pesticide concentrations (0.01 – 0.1 $\mu g\ L^{-1}$) is one of the major problems in Denmark over the last 15 years.

Distribution of pesticides in the environment is determined by two factors (i) physicochemical properties of the pesticides; and (ii) environmental factors. The pesticide concentrations are primarily the result of interacting dilution, sorption, transport and degradation processes. Degradation can involve both biotic and abiotic processes, where the microbial facilitated degradation (biodegradation) is particularly interesting, as it is the dominant route for the complete degradation of aromatic compounds to harmless inorganic products (Alexander, 1981). The degradative capacity of indigenous microbial populations in soil and subsurface environments is well known (Albrechtsen et al., 2001; Sørensen et al., 2003;

DeLipthay et al., 2003; Johnson et al., 2004; Buss et al., 2006), but it appears that low pesticide concentrations reduce both the rate and extent of degradation significantly even though competent degraders are present (Torång et al., 2003; Janniche et al., 2010). This phenomenon has been explained by the lack of expression of genes encoding catabolic enzymes or reduced microbial growth below a specific critical pesticide concentration. Detailed knowledge on the physiological bottlenecks that bring about such limiting concentrations is however unknown. Additionally, it remains to be elucidated what makes some degraders better at metabolising low contaminant concentrations compared to others and whether microbial populations adapted to metabolising low contaminant concentrations exists in natural aquifer environments.

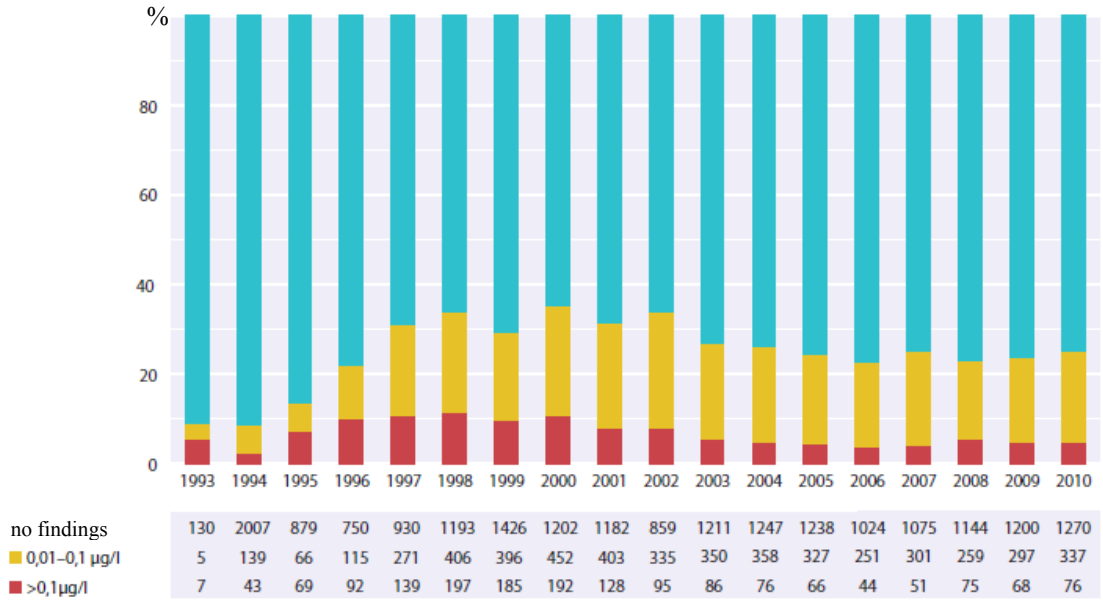

Figure 1.1. Distribution of pesticides in monitoring wells per year (1993-2010). The indicator does not contain the same wells from year to year, since these are analyzed in a rotation of up to five years. Number of wells in each of the three categories listed below each year.

Enrichment and isolation of bacterial strains has long been the method for selecting and isolating catabolic microorganisms (Caldwell et al., 1997; Dunbar et al., 1997) and it has been used successfully to obtain bacteria capable of degrading xenobiotic compounds (Pemberton et al., 1979; Itoh et al., 2000; Sørensen et al., 2005). High substrate concentrations are commonly used for isolation purposes (Topp et al., 2000; Zakaria et al., 2007; Breugelmans et al., 2007) and the obtained degraders may subsequently be used for bioaugmentation studies (Goldstein et al., 1985; Pham et al., 2009). Thus, populations existing in the same micro-niches which are adapted to metabolize low contaminant concentrations may have been overlooked although they could be more efficient

to remediate low levels of organic pollutants in contaminated environments. (Dunbar et al., 1997).

The **overall aim** of this thesis has been to improve our understanding of degradation of low pollutant concentrations in oligotrophic aquifers by developing and employing novel cultivation techniques to enrich and isolate microbial populations specially adapted to metabolize low concentrations of pollutants and by studying the mechanisms and bacterial physiology underlying the occurrence of low pollutant concentrations. This may lead to a better prediction of the fate of pollutants in subsurface environments and allow for remediation strategies aimed at designing more optimal conditions for degradation of pollutants at low concentrations *in situ*. The herbicide 4-chloro-2-methylphenoxyacetic acid (MCPA) was chosen as the model compound.

The thesis is based on three submitted manuscripts. The manuscripts are preceded by an introduction covering the relevant topics presented in the manuscripts, bringing further discussions in the study area. The discussion is supported by highlights from the manuscripts, when new accomplished knowledge could contribute to the discussion.

Paper I dealt with comparison of the metabolism, the physiological characteritics and the phylogenetic diversity of two 2-methyl-4-chlorophenoxyacetic acid (MCPA) degrading bacterial communities that were enriched from an aquifer on low or high substrate concentrations. In **Paper II,** we developed a novel approach, which combines long term cultivation with low diffusive fluxes, in attempt to isolate MCPA degraders specially adapted to low pollutant concentrations. **Paper III** dealt with the effect of substrate levels on cytometric characteristics of MCPA degrading strains during metabolic activity using flow cytometry, with a special focus on low nucleic acid (LNA) and high nucleic acid bacteria (HNA).

2. Life at low substrate concentrations

Most natural environments (e.g. marine waters, soil, and aquifers) are characterized by nutrient-poor (oligotrophic) conditions where available carbon concentrations are very low, usually in the range of 10 – 100 µg L^{-1} (Kjelleberg, 1993; Egli, 2010). This phenomenon restricts microbial growth in such ecosystems (Kirchmann, 1993; Morita, 1997). Until recently, microbiologists considered oligotrophic environments as "deserts" for life. Nevertheless, despite the low concentrations of available carbon compounds, bacterial communities have been shown to dominate these environments with densities of up to 10^5 – 10^6 living cells per mL (Whitman et al., 1998; Rappé et al., 2002). Today, we know that the microbes that are dominating these environments are perfectly alive, metabolizing and ready to grow when given the chance. Hence, they have developed strategies to cope with this situation (Egli, 2010). In this section, I will discuss the mechanisms behind the limitations of low concentrations and the adaptations that microorganisms developed to overcome these limitations.

2.1. Oligotrophy and oligotrophs

Oligotrophic environments are characterized by their limited resources of available carbon and energy (Kjelleberg, 1993). This phenomenon results from the fact that organic matter content is at low concentrations in these environments, and much of these organic matter is not readily available for microbes due to its chemical recalcitrance (van der Kooij et al., 1982; Morita, 1988). Physical barriers may also play a role in this context by blocking of microbes to the heterogeneously distributed nutrients (Smiles, 1988). Microorganisms have adapted and developed strategies by adjusting their cellular composition with respect to both structure and metabolic function in order to survive in such extreme environments (Poindexter, 1987). Such microorganisms that are evolutionary adapted to low substrate concentrations and low energy fluxes are known as oligotrophs (Semenov, 1991). Apart from their ability to use low substrate concentrations and to persist in chronic starvation, oligotrophs are also characterized by their inability to prosper in environments with high level of nutrients. However, there are contrasting reports regarding the ability of oligotrophs to grow at high nutrient concentrations. Some researchers define oligotrophic bacteria that are able to grow on media with low level of available carbon, as well as on media with a higher nutrient content (Kuznetsov et al. 1979). Ishida et al. (1986) called such organisms facultative oligotrophs, as opposed to obligate oligotrophs that cannot grow at high level of nutrients. These definitions used for oligotrophic organisms have been the subject of debate for

decades, nevertheless, it is widely accepted that the major characteristics of oligotrophic bacteria is the ability to grow in low nutrient media (0.5-15 mg of C L^{-1}), regardless of whether they grow in high nutrient media or not (Cho and Giovanni, 2004). In contrast to oligotrophs, microorganisms which are common in environments with greater nutritional opportunities are called copitrophs (Semenov, 1991; Koch, 2001).

Table 2.1. Physiological and genomic characteristics of oligtophic and copitrophic bacteria (modified from Lauro et al., 2009).

Physiological or genomic feature	Oligotroph	Copitroph
Growth strategy	Slow but consistent	Feast and famine
Cell size	Small (< 0.1μm^3)	Large (> 1μm^3)
Genome size	Small	Large
rRNA operon number	Few	Many
Growth rate dependence on media richness	No	Yes
Growing cells resistant to stress inducing agents	Yes	No
Starvation cross protection to high levels of stress inducing agents	Yes	No
Lag phase after starvation	No	Yes
Consistent cell yield during nutrient limited growth	Yes	No

Table 2.1 summarizes the physiological and genomic characteristics of oligotrophic and copiotrophic bacteria, based on two model strains; oligotroph *Sphingopyxis alaskensis* RB2256 and copiotroph *Photobacterium angustum* S14 (Lauro et al., 1999). As can be seen from the table, these two groups of bacteria use different growth strategies as a result of their different substrate requirements. Oligotrophs are assumed to be *K* strategists, while copitrophs are often *r* strategists (Watve et al., 2000). According to the theory, *K* strategists, in this case oligotrophs, grow slowly but consistently, both at low and high substrate concentrations. On the other hand, *r* strategists, copiotrophs, grow faster and more precipitous at high substrate concentrations. However, they may not survive or become dormant in environments that are deprived of readily accessible nutrients. These two groups of bacteria also differ in regards to their substrate uptake systems. Oligotrophic bacteria have high substrate affinity as evidenced by extremely low K_m and V_{max} values and adapt to low nutrient environments, while copiotrophic bacteria are characterized by having low substrate affinity for the substrate (Button, 1991). Therefore, oligotrophs have

competitive advantages over copiotrophs and in theory they could outcompete copitrophs at low substrate concentrations (Button, 1991, Hu et al., 1999). This hypothesis has been confirmed in natural soils by Hu and coworkers (1999), who showed that high carbon concentrations stimulated the growth of copiotrophic bacteria, while it has an inhibiting effect on oligotrophs. In contrast, at low carbon levels, copiotrophs halted growing whereas oligotrophs remain active, will gain advantage over copiotrophs, and will proliferate.

Oligotrophic bacteria are widespread in various natural ecosystems (Semenov, 1991). These bacteria are of great interest because they play a crucial role in the decomposition of organic matter and geochemical cycles of the elements in the environment. Their ability to grow at low nutrient concentrations makes them the key players in utilization of geochemically important, low molecular organic and inorganic substances or micro pollutants which occurs at very low concentrations (Semenov, 1991). Without the presence of oligotrophs such substances would reach significantly higher concentrations in the environment before copitrophs would begin to utilize them. In conclusion, oligtrophs are not only successive survivors in low nutrient ecosystems, but their activities are also responsible for maintaining the low concentration of nutrients.

To date, there are no standardized techniques or methods to detect oligotophic bacteria in natural environments, irrespective whether they are based on culture-dependent or independent approaches (Senechkin et al., 2010). Molecular markers identifying oligotrophic bacteria have been proposed (Luro et al., 2009), but not yet developed. Hence, oligotrophy remains to be a physiological rather than a taxonomic property, and can only be identified by cultural means so far (Senechkin et al., 2010). Expanding our knowledge on characteristics of oligotrophic bacteria such as phylogenetic, functional or physiological markers is very crucial in order to develop such tools. However, the only method for isolation and quantification of oligotrophic bacteria from natural ecosystems is the cultivation on media with very low concentrations of readily utilized carbon sources (Senechkin et al., 2010). Hence, the development of new methods and technologies is very important for the isolation of bacteria from oligotrophic environments. In **Paper II**, we attempted to isolate bacterial degraders, from an oligotrophic aquifer, that are specially adapted to low substrate concentrations. Indeed some of the isolates obtained in the study were most efficient at mineralizing low substrate concentrations and inhibited by higher concentrations, in line with the nutrient scarcity of their original habitat.

2.2. Biodegradation of pollutants at low concentrations

Every year about 300 million tons of synthetic compounds find their way into natural environments as a result of industrial and domestic use (Scharzenbach et al., 2006). Additional pollution comes from diffuse sources due to agricultural practice, in which several types of fertilizers and pesticides are applied each year (Koplin et al., 2000). The source, behavior, degradation mechanisms and treatment of pollutants occurring at high concentrations (mg L^{-1}) are relatively well understood, however it is more difficult to assess the effect of micropollutants that are commonly present in much lower concentrations (pg L^{-1} to μgL^{-1}) (Scharzenbach et al., 2006). Pesticide contamination in groundwater is an extensive pollution problem characterized by low chemical concentrations (Kolpin et al., 2000; Scheidleder et al., 1999). Biodegradation is the major route for the complete degradation of these organic pollutants to harmless inorganic compounds (Alexander, 1981), and much is known about the degradative capacities of microbial populations in the natural ecosystems. However, the microbial processes and degradation kinetics of organic chemicals at low, environmentally relevant concentrations can differ significantly from the processes at higher concentrations (Tros et al., 1996). Hence, our knowledge on these processes is mostly biased by the fact that the concentrations of organic chemicals used in the laboratory are far higher than those found in the environment.

The findings of low pollutant concentrations of organic pollutants in the environment, apart from many other environmental factors, may be the result of a physiological mechanism termed threshold concentrations. Alexander et al. (1999) defined threshold concentrations as 'the lowest concentration that sustains growth and represents the level below which a species brings about little or no chemical destruction'. A threshold concentration may present at different levels such as enzyme activity and induction of the enzyme synthesis or bacterial growth (Tros et al., 1996). According to the definition, when the concentration of a compound falls below a threshold value, the metabolism of the compound can be too slow to provide energy to the cells with at a rate needed for the maintenance of their metabolism (Button, 1995). Consequently, the cells cannot multiply and the population of bacterial degraders becomes too small to initiate the degradation of the compound (Roch and Alexander, 1997).

Threshold concentrations have been reported for pure and mixed microbial cultures (Pahm and Alexander, 1993; Roch and Alexander, 1997; Kovar et al.,

2002) as well as for environmental samples (Subba-Rao et al., 1982; Toräng et al., 2003). In a recent review by Egli (2010), dealing with growth and maintenance of microbial cultures at low concentrations, the threshold concentration for growth of pure cultures with a single substrate is suggested in the range of 1-100 µg L-1 depending on organism and carbon source. It was particularly Martin Alexander's research group who has reported threshold values for a number of organic compounds using microbial cultures (Pahm and Alexander, 1993; Roch and Alexander, 1997). In a study where the effect of low concentrations on the growth of four p-nitrophenol (PNP) degrading bacteria were studied, it has been shown that the PNP concentration does not increase the growth rates of the bacteria at concentrations less than 2-100 µg/L, when it was the sole added carbon source in culture, but it simulated growth at higher concentrations (Pahm and Alexander, 1993). The same study also revealed that the addition of other carbon sources to the media, such as glucose, decreased the threshold concentrations to lower values, suggesting that microorganisms may thus be able to mineralize substrates in natural waters at concentrations below those suggested in laboratory experiments.

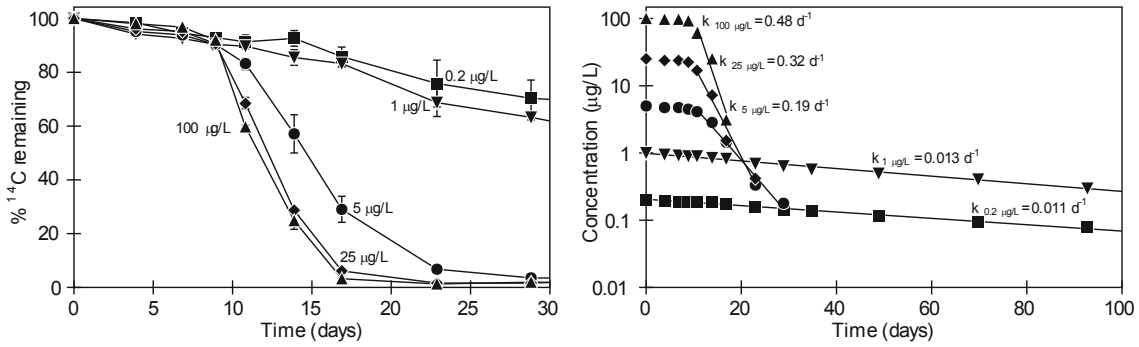

Figure 2.1. Degradation of 2,4-D in aquifer sediment + groundwater at different initial concentrations given as % 14C remaining (left) and concentration (rigth). 1st order kinetics were used to calculate degradation rates (reproduced from Toräng et al., 2003).

The threshold phenomenon has also been studied with natural samples. Toräng et al. (2003) studied the degradation kinetics of the two phenoxy acids, methylchlorophenoxypropionic acid (MCPP) and 2,4-dichlorophenoxy acetic acid (2,4-D) in sediment and groundwater samples at low concentrations (0.2-100 µg L^{-1}). Below a concentration of 1-10 µg L^{-1}, the degradation follows non-growth kinetics, while above a threshold value the biodegradation rate accelerated gradually due to selective growth of specific biomass, measured by most probable number method. Figure 2.1 shows the degradation of 2,4-D at different concentrations (Toräng et al., 2003) and it can be seen that growth-linked degradation kinetics with biphasic curves shifts to non-growth kinetics

below 1 μg L^{-1}. Furthermore, threshold values showed differences between the two phenoxy acids, indicating that even compounds having very similar structures may have different threshold values.

The above mentioned examples demonstrate the existence of threshold concentrations various compounds. However, significance and relevance of threshold concentration in natural ecosystems lacks more experimental evidence and await many unanswered questions. Firstly, it should be pointed out that threshold concentrations for growth-linked biodegradation are not fixed values, but vary between different contaminants and microorganisms (Rapp and Timmis, 1999). Furthermore, threshold concentration may also vary according to the physiochemical properties and composition of the environment itself. Several studies reported that simultaneous utilization of alternative carbon substrates reduces threshold concentrations for induction, utilization and growth of microbial cultures (Pahm and Alexander, 1993; Kovar et al., 2002). This suggests that threshold value of a given compound under laboratory conditions may not exhibit the threshold in samples of natural environment. Another factor that may cause variation of the threshold of a compound is the microorganisms. It is a well known fact that energy requirements and maintenance vary among microorganisms (see section 2.1), thus threshold values differs among the species (Schmidt et al., 1987; Pahm and Alexander, 1993). These differences in threshold concentrations for growth suggest that selecting microorganisms with higher affinities to the contaminant may be a key feature for bioremediation of micropollutants. This requires development of new cultivation techniques aimed at selecting oligotrophic bacteria (**Paper I and II**) and expanding our knowledge on the physiological features that makes this group of bacteria better at mineralizing organic contaminants at low concentrations (**Paper I and III**).

3. Unravelling bottlenecks for degradation of micropollutants

3.1. Isolation of contaminant degrading bacteria

Isolation of bacteria with the ability to degrade organic contaminants has been carried out for the last five decades (Stainer et al., 1966). Even though, recent advances in culture independent molecular approaches have improved knowledge of microbial ecosystems, isolation of bacterial species in pure culture remains to be the only way to fully characterize them, both for their physiological and catabolic properties. Culture-dependent methods are therefore still a powerful tool that can be used in conjunction with molecular ecological methods to investigate the functions of many as-yet-uncultivated bacteria that will lead to a better understanding of the microbial ecosystems.

3.1.1. Effect of substrate concentrations on enrichment culturing

Enrichment culturing has long been the method of choice for selecting and isolating catabolic microorganisms (Caldwell et al., 1997; Dunbar et al., 1997), and it has been used successfully to obtain bacteria capable of degrading xenobiotic compounds (Pemberton et al., 1979; Konopka, 1993; Itoh et al., 2000; Topp et al., 2000). The enrichment process is generally the inoculation of a source of bacteria into a defined mineral media, supplemented with the contaminant of interest as the sole source carbon (**Paper I**), nitrogen (Topp et al., 2000), both carbon and nitrogen (Sørensen et al., 2005), sulfur (Furuya et al., 2001) or phosphorus (Balthazor and Hallas, 1986). The theory is that only the bacteria metabolizing the compound of interest will grow significantly and outgrow the rest of the bacteria in the inoculum. Enrichment cultures are generally plated onto solid media, which typically consist of an agar-supplemented mineral media with the contaminant as an essential nutrient source, in order to provide pure cultures. However, it is generally unclear whether the isolated strains originating from the enrichment cultures are representative of active and dominant contaminant-degrading bacteria in the environment or merely an artifact of the enrichment procedure (Bollmann et al., 2010). Dunbar et al. (1997) reported a significant lower diversity of 2,4-dichlorophenoxy acetic acid (2,4-D) degrading bacteria obtained from enrichment cultures, compared to the diversity of bacteria obtained by direct plating of soil on 2,4-D-containing agar plates. The diversity of the catabolic genes showed the same pattern between the two groups of isolates (Dunbar et al., 1997). However one should keep in mind that the use of direct plating for isolating degraders may have

drawbacks, such as over-growing of bacteria that are not able to degrade the contaminant. Furthermore, these authors did not study the catabolic genes in soil before the cultivation of the bacteria, therefore it is not certain that the direct plating technique resulted in a loss of diversity compared to the total diversity present in soil. Another limitation of the enrichment culturing is the tendency to select for fast-growing bacteria that might outcompete the slow growing bacteria (Pace, 1997). Due to the fact that high substrate concentrations are commonly used during isolation processes (Topp et al., 2000; Zakaria et al., 2007), bacterial populations existing in the same micro-environments that are naturally adapted to metabolizing substrates at low concentrations may be overlooked (Dunbar et al., 1997). Apart from selecting irrelevant populations, use of high pollutant concentrations in cultivation of microorganism from oligotrophic environments, may inhibit the growth of bacterial cells (Postgate and Hunter, 1964). Indeed, we have shown that high MCPA concentrations had a negative effect on the growth of MCPA degrading bacteria on agar plates and the number of colonies decreased with the increasing MCPA concentrations (**Paper II**).

One way to demonstrate the effect of substrate concentrations during enrichment processes could be comparison of bacterial communities obtained by using different substrate concentrations. In **Paper I,** we have compared various characteristics of two bacterial cultures obtained by employing a conventional enrichment approach based on different MCPA concentrations originated from the same sediment material. These populations showed differences in metabolic efficiency and community composition as well as physiology at the single cell level. The bacterial cultures selected on low MCPA concentrations were more effective in mineralizing both low and high MCPA concentrations in comparison to those enriched with high MCPA concentrations. Furthermore, the pure bacterial strains isolated using these enrichments also showed differences in metabolic efficiencies and diversity of catabolic genes. The bacterial strains that were isolated from enrichment cultures selected on low MCPA concentrations were better at mineralizing the compound at environmentally relevant concentrations than the ones selected on high concentrations of MCPA (**Paper II**). These findings strengthen the importance of the pollutant concentrations as a determining factor used in the enrichment procedure and suggest that using environmentally relevant substrate concentrations, in contrast to the classical high concentrations, would provide bacterial communities that are more efficient in terms of metabolic functionality and stability when degradation of low pollutant concentrations is desired.

3.1.2. Improving the culturability: Novel cultivation approaches

Only a small fraction of the existing microbial diversity has been cultivated till date and more than half of the bacterial phyla still do not have cultured representatives (Hugenholtz et al., 1998; Keller and Zengler, 2004; Rappe and Giovanni, 2003). Culture-independent analyses revealed that <1% of total bacterial species can be recovered using standard laboratory cultivation methods (Amann et al., 1995; Leadbetter, 2003) and this minor proportion of culturable bacteria do not reflect the functional and phylogenetic diversity present within any environment (Hugenholtz et al., 1998). The large gap between what we know about the cultured microbial diversity versus uncultured members of these communities is illustrated in Figure 3.1. The fraction of culturable members in regards to the total members differs in each prokaryotic group. Accessing this uncultured portion is of substantial basic and applied importance, especially for unraveling the roles of these species in key processes of bioremediation, such as biosorption, bioaccumulation, biotransformation, and biodegradation (Bollmann et al., 2010). The knowledge about uncultured microbial diversity has risen dramatically over the last two decades by culture independent analysis (Rappe and Giovanni., 2003). These methods are ideal to get an overview of the total species diversity of a microbial community and to explore the diversity of functional genes (e.g., *tfdA*) among the members of the community. However, it is only through isolation of individual bacterial species in pure cultures that a detailed characterization of the physiology and genome may be undertaken. Therefore, cultivation-dependent approaches are still a powerful tool that can be used in conjunction with molecular methods to investigate the functions of many as-yet-uncultivated bacteria.

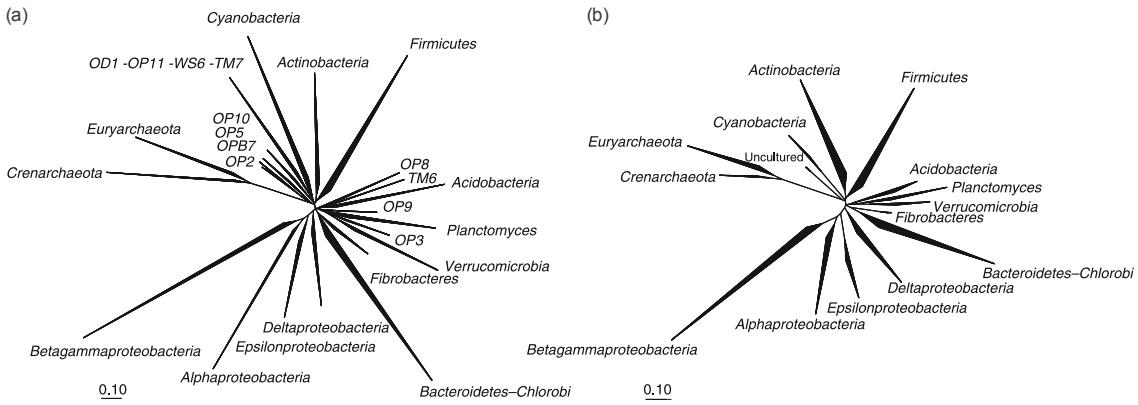

Figure 3.1. Phylogenetic relationship between selected prokaryotic groups represented by 16S rRNA gene sequences of total (culturable and uncultured) bacteria (a) and of culturable-type strains only (b). Bars indicate a similarity distance of 10% between sequences (da Rocha et al., 2009).

The lack of success in isolating pure bacterial cultures from the environment may be due to several reasons. These include specific requirements of certain bacteria such as nutrients, pH conditions, incubation temperature, oxygen level and lack of certain growth factors and signaling compounds (Kopke et al., 2005; Vartouikan et al., 2010). Use of high substrate concentrations, which is a bias and a limiting factor in cultivation studies, was discussed detailed in section 3.1. Table 3.1 summarizes the factors that limit bacterial isolation from natural environments and proposed approaches to overcome those problems.

Table 3.1. Factors limiting bacterial isolation from natural environments and proposed approaches to improve culturability (modified from da Rocha et al., 2009).

Factors that limit growth of the uncultured bacteria	Approaches to improve culturability	Environments from where novel isolates were obtained	References
Inability to grow at high substrate concentrations and overgrowth by faster growers	●Reducing nutrient availability in growth medium ●Applying extended incubation times	●Bulk soil ●Seawater	Janssen et al., 2002; Sait et al., 2002; Davis et al., 2005 Rappe et al., 2002
Media selectiveness for particular groups of microorganisms	●Developing of media that select for a different or a broader spectrum of microorganisms	●Bulk soil	Joseph et al., 2003; Davis et al., 2005
Compounds inhibiting bacterial growth	●Diffusion or dilution of growth-inhibiting compounds, (i.e; use of diffusion chambers) ●Application of alternative solidifying agents	●Marine sediment ●Fresh water sediment	Bollmann et al., 2007 Tamaki et al., 2005
Syntrophic growth or requirement for growth factors produced by other microorganisms	●Use of diffusion chambers ●Addition of syntrophic (helping) bacteria in enrichment cultures	●Rice paddy field ●Fresh water sediment	Sakai et al., 2007 Bollmann et al., 2007
Low abundance in environmental samples	●Single-cell detection combined with parallel microbial cultivation ●High-throughput dilution to extinction cultivation method	●Bulk soil ●Seawater	Zengler et al., 2005 Rappe et al., 2002
Formation of colonies that are undetectable by the unarmed eye	●Colony identification by FISH in microtitre plates ●Colony identification by hybridization on nylon membranes ●Microcolony cultivation	●Bulk soil ●Sea water	Stevenson et al., 2004; Ferrari et al., 2005 Rappe et al, 2002

Recently, significant efforts have been made to improve microbial recovery by application of novel cultivation approaches (Table 3.1). These include cultivation with a diffusion growth chamber (Kaeberlein et al., 2002), high-throughput culturing by dilution-to-extinction (Connon and Giovanni, 2002; Rappe et al., 2002), filtration methods (Wang et al., 2009), single cell encapsulation (Zengler et al., 2002), use of micromanipulators and optical laser tweezers (Frochlich and Konig, 2000) and modified plating methods including addition of signaling compounds, using alternative gelling agents, increasing incubation times, and *in situ* cultivation (Eilers et al., 2001; Jannsen et al., 2002; Kalyuzhnaya et al., 2008; Davis et al., 2005; Aoi et al., 2009). Cultivation methods based on modified traditional approaches have been successfully used to isolate previously uncultured, phylogenetically distinct bacteria. To date, the majority of culture media are nutrient-rich. These conditions may favor the growth of fast growers at the expense of slow growers and also mask rare species (Koch, 1997). Additionally, they may inhibit the growth of cells that are adapted to nutrient-poor conditions. To overcome this bias, use of dilute nutrient media was used successfully by many researchers to isolate previously unculturable bacteria (Connon and Giovannoni, 2002; Rappe et al., 2002; Zengler et al., 2002, Jannsen et al., 2002). Another modification applied is the use of extended incubation times. Davis et al. (2005) reported that prolonged incubation times up to 12 weeks has revealed increasing colony counts and an increased recovery of rarely isolated strains with time. Similarly, strains from the SAR11 clade were isolated by increasing incubation period up to 8 months (Song et al., 2009).

An alternative approach to culture previously uncultivated bacteria is to mimic the natural conditions through cultivation process. Diffusion chambers, that allow the passage of substances from the natural environment across a membrane, has been used by several authors for growing bacteria that were previously uncultivated (Kaeberlein et al., 2002; Nichols et al., 2008; Bollmann et al., 2007). However the method was not always selective enough to obtain pure cultures since the obtained bacteria grew only in the presence of other bacteria when cultured on solid media (Kaeberlein et al., 2002). Another technique mimicking the natural conditions was the use of sterile fresh- (Wang et al., 2009) and marine- (Rappe et al., 2002) waters for the culture of as-yet-uncultivated organisms. Recently, Wang et al. (2009) have isolated and characterized the smallest free-living heterotrophic organisms known in culture by using sterile freshwaters following dilution of samples and separation of indigenous bacterial communities by filtration and fluorescence-activated cell sorting. Yet another technique was introduced by Ferrari et al. (2005) that includes a polycarbonate

membrane support and soil extract as a substrate. The system was useful to cultivate novel bacterial species, but bacteria remained part of a mixed community on the membrane, with the consequent difficulty in isolation of individual microcolonies for further characterization. The method was later developed by combining it with fluorescence viability staining and advanced micromanipulation for targeted isolation of viable, microcolony-forming soil bacteria (Ferrari and Gillings, 2009).

3.1.3. Isolation of Bacterial Degraders by LFF Plates

Most of the approaches explained above share one basic strategy which is the use of growth conditions mimicking the chemical and physical properties of the natural habitat. In this PhD study, we have followed this basic strategy and developed a novel plate-based cultivation method, called Low Flux Filter (LFF) plates, to isolate herbicide-degrading bacteria from an oligotrophic aquifer. This approach involves direct isolation where a low flux of substrate is diffusing to cells growing on a membrane placed on an agar surface during extended incubation times (**Paper II**) (Figure 3.2 and 3.3). The isolation approach used herein has three important components;

1. Use of a polycarbonate membrane: It is a well known fact that the solidifying agar contains several utilizable sugars and amino acids in high concentrations that can be used by bacteria (Schut et al., 1993) and causes non-specific growth. One important advantage of the membrane system is to bypass this problem since the membrane creates a physical barrier between the growing bacteria and the agar medium. Secondly, the membrane allows the diffusion of the substrate to the cells. This is particularly important when the aim is to isolate slow growing bacteria. It has been demonstrated earlier that low diffusive flux of substrates increases the relative fitness of slow growing bacteria competing with relatively faster growing bacteria (Dechesne et al., 2008).

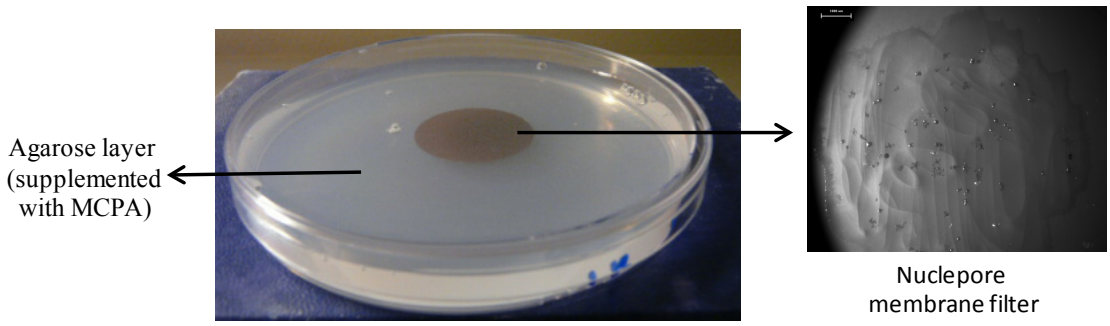

Figure 3.2. Representation of the LFF plate system used in this study (Paper II).

2. Use of low substrate concentrations: It has been discussed earlier that the high concentrations used in traditional cultivation approaches has major disadvantages such as inhibiting growth and outcompeting slow growing organisms. Thus, we have used relatively lower substrate concentrations in the study compared to traditional approaches. We have observed decreasing number of colonies on agar plates with increasing substrate concentrations. Moreover, inocula used in the plates were either obtained by directly extracting the biomass from the sediments or by enriching the herbicide-degrading populations on low (100 μg L^{-1}) or high (25 mg L^{-1}) substrate concentrations. The direct extraction bypass the possible biases of the enrichment process and relatively lower concentrations used in the enrichment process enables growth of slow-growing bacteria.

3. Extended incubation time: Prolonged incubation times up to 4 months were used during isolation process by transferring the membranes onto fresh plates. Incubation times on the order of months are only rarely used, and are generally in the range of 1 week to 1 month. Davis et al. (2005) reported increased incubation time results in increased viable cells on media with low substrate concentrations. A similar continuous-cultivation approach has been successfully used by Rasmussen et al. (2008) for isolating mercury-resistant bacteria from a subsurface environment, where the authors reported that the diversity of bacteria growing on the membranes increases with the transfer of polycarbonate filters to a fresh medium. This phenomenon can be explained by the depletion of nutrients, at the medium-membrane interface, limiting the growth of bacteria on the membranes. The advantage of applying a continuous-cultivation approach has also been suggested by Bollmann et al. (2007) who demonstrated that continuous cultivation may adapt some microorganisms for growth under otherwise prohibitive in vitro conditions.

In our study, we have also used standard MCPA plates that are devoid of the membrane filter in parallel to LFF plates, and none of the strains that were isolated by these plates showed MCPA mineralizing capacity after the cultivation. This clearly indicates the importance of physical separation of growing bacteria and the agar medium and of extended incubation periods with continuous supply of the substrate.

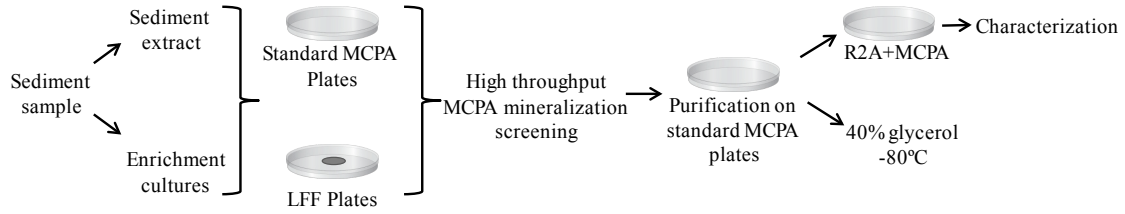

Figure 3.3. Overall scheme of the isolation process used in this study (Paper II).

Our novel method led to the isolation of five different MCPA-degrading strains. These strains, the first MCPA-degraders to have been directly isolated on MCPA, belonged diverse Proteobacteria genera: *Achromobacter*, *Pseudomonas*, *Variovorax*, *Cupriavidus*, and *Sphingomonas*. In line with the nutrient scarcity of their original habitat, the isolates were most efficient at mineralizing low MCPA concentrations. That shows that our method is efficient at providing bacterial isolates that are relevant when degradation of low pollutant concentrations is desired, in contrast to conventional isolation methods that use complex media and high substrate concentrations, resulting in biased selection of copiotrohic organisms.

3.2. Applications of flow cytometry in environmental microbiology

Only a small percentage of microorganisms are cultivable with the currently used culturing methods (Rappe and Giovanni, 2003). Despite the recent advances and new methods developed, many cultivation attempts fail. This is due to the fact that we still do not know how to provide the necessary conditions to grow microorganisms in the laboratory (Tyson and Banfield, 2005). There is an emerging need to find new ways to isolate microorganisms from their natural environments. Information about genetic potential and physiological characteristics of a microorganism obtained by cultivation-independent can be used to predict its metabolic and nutritional requirements. Hence, this could provide the information to overcome the bottlenecks that prevents the cultivation of many microorganisms (Tyson and Banfield, 2005). Flow cytometry is one of the approaches among many others to reach this target. Hence, in this section I will try to address the recent developments in this field and discuss how flow cytometric studies can be used to access the missing microbial diversity.

3.2.1. Flow cytometry, overview and applications

Flow cytometry (FCM) is a routine technique used in cellular biology for examining and sorting microscopic pPapers. It allows simultaneous multiparametic analysis of the characteristics of single cells (i.e size, shape and nucleic acid content), regarding each cell's light scattering and/or fluorescence properties, as they flow through an optical and/or electronic detection apparatus. The obtained data is then converted into digital information enabling population to be closely monitored (Figure 3.4).

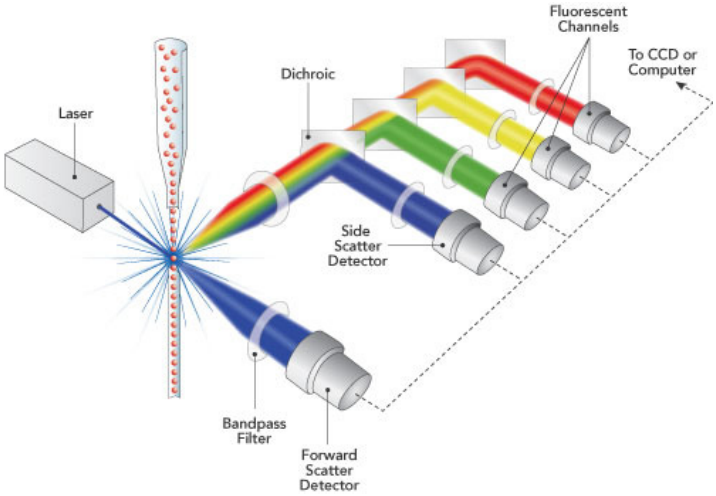

Figure 3.4. An illustration of the flow cytometry; cells are focused in a sheath liquid, and then hit by the laser beams individually. Forward and side scattered light are detected by photomultipliers, as (via suitable filters) fluorescence, and are collected and quantified by a computer. From: http://m.semrock.com/flow-cytometry.aspx.

FCM has been used primarily in medical applications, while its application on prokaryotic cells has been hitherto rather limited mainly because of the difficulty to interpret signals from very small objects. However, the recent technical advances and the development of sensitive fluorescent nucleic acid stains have led the intensive use of FCM in environmental microbiology (Czechowska et al., 2008). This attraction causes from the facts that FCM is fast (<3 min to measure a sample), accurate (<5% instrument error), sensitive (detects down to 100 cells per mL), allows generation of multiple parameters (Egli, 2010; Wang et al., 2010). Another big advantage of FCM is that it can detect microbial cells irrespective of their culturability, which overcomes a major bottleneck in the field of microbiology. Furthermore, with the possibility of cell sorting, it supplies information at the single cell level (Wang et al., 2010). On the other hand, major limitations of FCM are its sophisticated and complex data validation and that it is restricted to liquid sample analysis. Nevertheless, soil and sediment samples can

be processed with pretreatment techniques such as sonication, permeablization or separation of cells prior to analysis. There is a wide range of useful laboratory based research applications of FCM in conjunction with several different fluorescence stains, including enumeration of bacterial cell concentration (Lebaron et al., 1998), characterization of bacterial growth (**Paper I**), community structure analysis, characterization of different physiological states of bacteria at the single cell level (Nebe-von- Caron et al. 2000), and assessment of approximate cell size (Felip et al., 2007). Figure 3.5 shows of the different steps for FCM analysis of environmental samples (Wang et al., 2010).

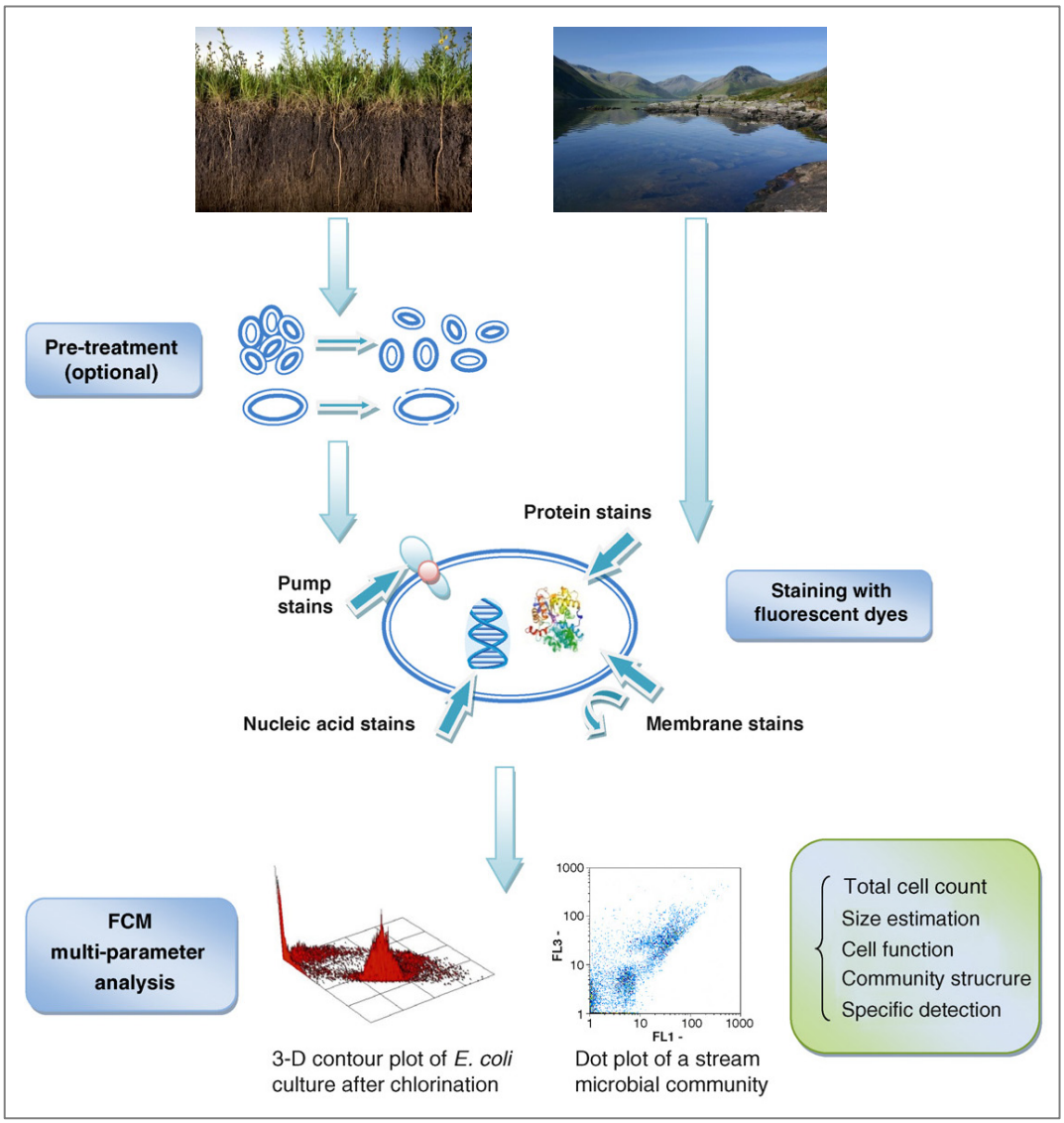

Figure 3.5. Schematic overview of the different steps needed for FCM analysis. The top two images represent soil (left) and freshwater environments (right). Various components of the cell can then be labelled with dyes for intracellular (CFDA) and membrane proteins (2-NBDG), nucleic acids (SYBR, SYTO) and the cell membrane (bis-oxonol) (modified from Wang et al., 2010).

Flow cytometry can also be used in combination with cultivation approaches. A very well known example is the single cell encapsulation developed by Zengler et al. (2002). This technique uses microcapsules to encapsulate single cells combined with parallel microbial cultivation under low substrate flux conditions, which is followed by flow cytometry analysis to detect the microdroplets that contain microbial colonies. Another example to such a combined approach is the isolation of very small bacteria (<0.05 mm^3) from freshwater by Wang et al. (2009). In this study, the researchers used a cultivation approach based on very low substrate concentrations that is coupled with FCM. This approach has led to the isolation of the smallest free living bacteria in culture.

3.2.2. Flow cytometry as a tool to analyze single cell physiology

One promising application of FCM is the differentiation and characterization of physiological activity of microbes at the single cell level. This is possible when FCM is used together with two or more fluorescent stains simultaneously that measure parameters such as nucleic acid content, enzyme activity, pump activity, respiration rates, and cellular membrane integrity (Nebe-von- Caron et al., 2000; Joux et al., 2000; Berney et al., 2007). One widely used approach is the differentiation of live and dead cells by double staining with the nucleic acid binding SYTO or SYBR Green dyes (green fluorescence) and propidium iodide (PI) (red fluorescence) (Berney et al., 2007; **Paper I and III**). In this method green fluorescing dye can enter all cells, whereas only dead cells are permeable to red fluorescing PI. In addition to live/dead differentiation, FCM allows to discriminate various intermediate states of microorganisms (Nebe-von- Caron et al., 2000). For example, a cell can be active and capable of replicating while some may be active but not able to replicate. It could as well be that a cell is not active because it is injured or damaged. Such differentiations are especially relevant for the assessing the growth and physiological activity of microbes in oligotrophic environments, since conventional plating techniques fail to do so (Wang et al., 2010; Czechowska et al., 2008).

3.2.3. Low nucleic acid bacteria: Underestimated populations

One of the most interesting findings that have emerged from the flow cytometric studies is the fractioning of bacterial cells into two major fractions: high-nucleic acid (HNA) content bacteria and low-nucleic acid (LNA) content bacteria (Gasol et al., 1999; Lebaron et al., 2001; Felip et al., 2007). This fractioning is based on differences in the individual cell fluorescence (related to the nucleic acid content) and in the side and forward light scatter signal (related to cellular size). Although

the occurrence of this distribution is widely accepted, there are many contrasting reports in the literature regarding the physiological, ecological and functional relevance of these fractions (Lebaron et al., 2002; Jochem et al., 2004). The most accepted theory was that HNA-bacteria represent the active part of the community and LNA-bacteria are inactive or dead cells (Lebaron et al., 2001; Lebaron et al., 2002; Servais et al., 2003). However, recent findings showed that LNA-bacteria is an active part of microbial communities having equal or even faster growth rates than the HNA-bacteria (Zubkov et al., 2001; Jochem et al., 2004; Wang et al., 2009; **Paper I**). However, to my knowledge, no study has reported a direct a link between the growth of LNA bacteria and the specific metabolic activity. In **Paper I**, we have shown that growth of LNA-bacteria coincided with metabolic activity in MCPA degrading cultures enriched with low levels of substrate (Figure 3.6). However this pattern was not observed with cultures enriched with high substrate concentrations. These findings lead to two important conclusions (i) LNA bacteria are active and they might be highly overlooked due to the fact that most cultivation approaches use high levels of substrate to select microorganisms from the environment. (ii) These underestimated populations could play a role in degradation of low pollutant concentrations in contaminated environments.

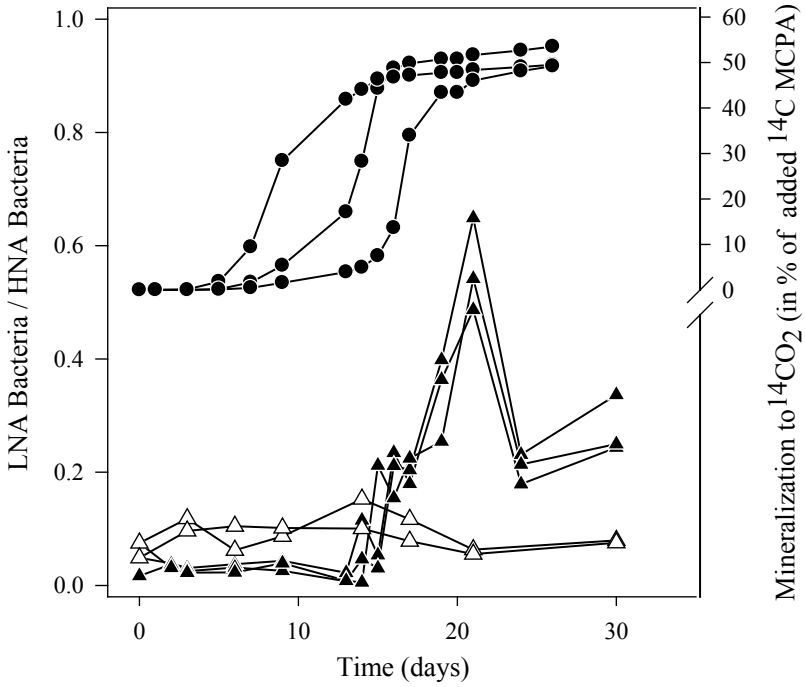

Figure 3.6. Mineralization of ^{14}C-labeled MCPA to $^{14}CO_2$ (closed circles) and the ratio of LNA- to HNA-bacteria (closed triangles) in the enrichment cultures. The open triangles indicate the control microcosms without pesticide addition. These data are shown individually for triplicate samples. The MCPA concentration used in the experiment is 100 µg L^{-1} (Paper I).

The recent knowledge on LNA bacteria and their activity in various environmets brings forth an important question: Are LNA-bacteria a different physiological state of HNA cells, or do they represent a unique group of organisms? The general acceptance of the HNA- and LNA-bacteria as an active versus dichotomy is overly simplistic to explain such a complex question. Bouvier et al. (2007) suggested four different scenarios regarding the nature of these fractions, and of the interactions that exist between them. These four scenarios are illustrated in Figure 3.7 (i) HNA are active cells while LNA fraction consists of inactive, injured or dead cells originating from HNA cells. (ii) LNA fraction is composed of cells with different physiological states (i.e active, inactive, dormant and injured cells), whereas HNA cells originate from active LNA cells that are undergoing cell division (iii) LNA and HNA fractions are different communities with their own characteristics. (iv) These two fractions are the result of complex processes that could include both dynamic exchanges between the LNA and HNA cells, as well as characteristics that are distinct to each of the fractions.

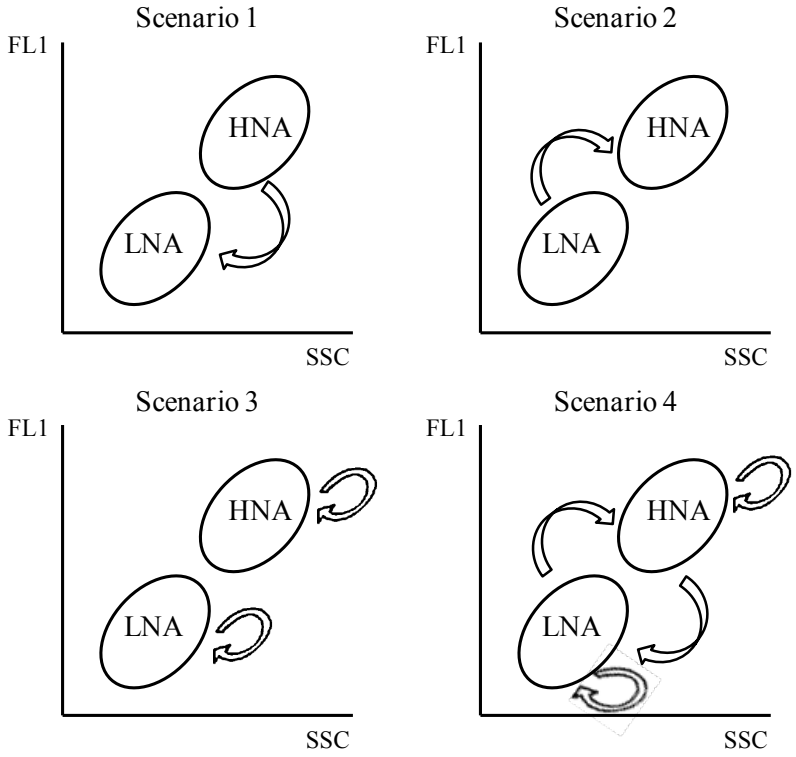

Figure 3.7. Illustrated outline of the four scenarios that may be envisioned regarding the nature of LNA and HNA groups of bacterial cells. Green fluorescence (FL1) is used as an indicator of apparent cellular nucleic-acid content, and sideward scatter signals (SSC) as an indication of cellular size (reproduced from Bouvier et al., 2007).

In **Paper III**, we performed flow cytometric analyses of three different pure MCPA degrading bacterial strains to determine the physiological characteristics in response to different substrate concentrations, in order to get a better understanding of the physiological, ecological and functional relevance of LNA-HNA populations. We have shown that strains isolated from sediment material without foregoing MCPA addition (strain ERG1) and from cultures enriched with low levels of MCPA (strain ERG2) harboured LNA cells. The dominancy of LNA cells decreased with increasing MCPA concentrations, indicating that substrate concentration has an effect on LNA harbouring populations, shifting them to HNA fraction. On the other hand, bacterial strain isolated from cultures enriched with high MCPA concentrations (strain ERG5) had strict HNA properties regardless of the concentrations used in the study (Figure 3.8).

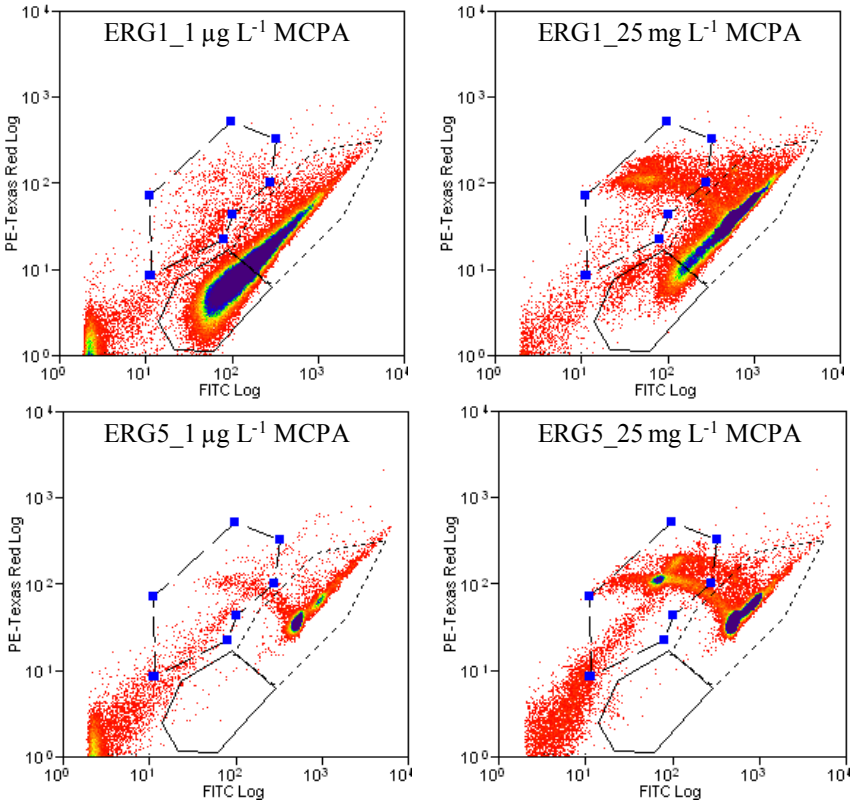

Figure 3.8. Flow cytometric dot plots of the tested bacterial strains in relation to the MCPA concentration. The solid line indicates LNA bacteria, the dotted lines indicate HNA bacteria and dashed lines indicates dead cells.

We have shown that high MCPA concentrations have an inhibiting effect on ERG1 and ERG2 strains (**Paper II**). The inhibition at high substrate concentrations and the dominancy of LNA bacteria in these strains, particularly at low MCPA concentrations, supports the findings of Li et al. (1995) who

reported that LNA-bacteria are better adapted to oligotrophic conditions and that their dominance decreases with nutrient input in aquatic ecosystems. Furthermore, our results are in line with the intermediate scenario (scenario 4) discussed above that explains this bimodal distribution as a result of both the passage of cells from one fraction to the other as well as the existence of bacterial groups that are characteristic of either LNA and HNA fractions. The mechanism beneath the passage of cells from one fraction to the other may be due to several reasons such as DNA replication and reduction during cell division (Lebaron and Joux, 1994), increase in DNA content during metabolic activity (Gasol et al., 1999) or adaptive changes in genome size over extremely short time-scales (Nilsson et al., 2005).

Regardless of the underlying mechanisms, the existence of LNA and HNA fractions in various environments, which are populated by entirely different phylogenetic group of bacteria having different physiological characteristics suggest that this bimodal distribution plays a key role in the functioning of bacterial communities. The findings in the scope of this thesis further indicate that LNA-bacteria are active and dominate the population when substrate concentrations are in low levels. Moreover, this group of bacteria may be overlooked through laboratory experiments and they may play important roles of remediating low contaminant concentrations.

4. Phenoxy Acids

The phenoxy acid (PA) herbicides are a subgroup of the larger group phenoxy herbicides, that consist more than 50 different compounds (Wood, 2004). PA herbicides have been intensively used for agricultural purposes throughout the world since their development in the 1940s for the control of broad leaf weeds. They were designed specifically to mimic the function of the plant growth hormone auxin, and stimulate the plant to overgrow and finally cause its death (Cremlyn, 1991). In Denmark, the use of these herbicides has been considerably restricted for the last couple of years by the Danish Environmental Protection Agency (Miljøsttrelsen. 2011). However, the previous heavy use and the weak retention of the compound have resulted in contamination of the groundwater, and they are still being detected in the groundwater in concentrations exceeding the European Commision drinking water limits (EU 2011; GEUS 2011) (Table 4.1). Concentrations of PA herbicides reported for groundwater are in the range of 0.1-6.0 µg L^{-1} (Felding et al., 1995; GEUS., 2011), indicating that contamination of PA herbicides in groundwater is a pollution problem characterized by low chemical concentrations.

Table 4.1. The most frequently detected pesticides and pesticide metabolites in the in the groundwater monitoring program (1990 - 2010), active wells (1992 - 2010) and in 'other wells' (1990 - 2010) The compounds are ranged due to percentage of detections. The category 'other wells' includes abandoned wells, self-control performed by certain water works and by private wells (modified from GEUS 2011).

Moni-toring prog-ram	Groundwater monitoring			Control of wells			Other wells		
Ran-king	Compound	% detec -tion	% above thres-hold	Compound	% detec-tion	% above thres-hold	Compound	% detec -tion	% above thres-hold
1	BAM	21.1	8.4	BAM	19.6	4.3	BAM	30.0	14.3
2	DEIA	14.6	4.1	4-Nitro-phenol	3.2	0.0	4-Nitro-phenol	9.2	5.9
3	Atrazine, deisopropyl	10.9	1.8	Bentazone	2.7	0.4	DEIA	8.9	1.4
4	4-Nitro-phenol	9.5	0.6	4CPP	2.6	0.4	Atrazine, deethyl-	7.1	1.6
5	Atrazine, deethyl	8.4	1.5	Mecoprop	2.5	0.1	Atrazine, deisopropyl	6.8	1.4
18	4CPP	2.8	0.9	**MCPA**	0.7	0.1	2,6-dichlore-benzosyre	2.6	0.2
19	**MCPA**	2.4	0.4	Atrazine, hydroxyl-	0.5	0.0	Hexazinon	2.2	0.7

The degradation of phenoxy herbicides like 4-chloro-2-methylphenoxyacetic acid (MCPA), 2,4-dichlorophenoxyacetic acid (2,4-D), and related compounds has been studied intensively over the last 50 years, with the majority of the work being done on 2,4-D. These studies have revealed detailed knowledge about the degradation and mineralization kinetics, the metabolic pathways, the genes and enzymatic systems involved in the degradation, and the isolated microbial degraders. Hereby, I will discuss these topics separately with the special focus on MCPA which has been used as a model compound in this study.

4.1. Physical and chemical characteristics of the phenoxy acids

The phenoxy acids are weak organic acids having a high water solubility and low sorption to the soil. These properties make these compounds very mobile in soil, thus susceptible to leaching from soil to water bodies. The contamination of drinking water resources with these compounds has been reported several times (Brusch and Juhler, 2003; GEUS 2011). The structure of the compounds in this group is characterized by a dichloro-, or chloro-methyl-substituted aromatic ring either with a propionic acid group (mecoprop, dichlorprop), or an acetic acid group (MCPA, 2,4-D) coupled to the ring through an ether bond (Table 2.1).

4.2. Biodegradation of phenoxy acids in aquifers

Microbial degradation of phenoxy acids is reported as the most important process of removal of phenoxy acids from the environment (Alexander, 1981). In soils frequently exposed to phenoxy acids, half life values of less than 30 days are typically reported (Bælum et al., 2008b; Gonod et al., 2006). The degradation of phenoxy acids in aquifers and sediments has been reported several times in the literature (Larsen et al., 2000; Tuxen et al., 2000; Broholm et al., 2001; de Lipthay et al., 2003; Sørensen et al., 2006). deLipthay et al. (2003) indicated that subsurface environments were able to adapt phenoxyacid degradation following exposure to even low herbicide concentrations. However, phenoxy acids have been reported as being more recalcitrant in these environmental matrixes (Johnson et al, 2000; Pedersen, 2000). This phenomenon has previously been related to several interacting factors such as limited availability of organic and inorganic nutrients (Veeh et al., 1996), low amount of biomass and microbial activity (Bælum et al., 2008b) and reduced bioavailability of the pesticide due to sorption (Jensen et al., 2004). In all reported experiments lag phases of various lengths were observed prior to degradation, possibly due to several reasons such

as induction of enzyme activity, horizontal gene transfer and growth of specific degraders (van der Meer et al., 1992; Sørensen et al., 2006). Similar findings were observed in this thesis (**Paper II**).

4.3. Metabolic pathway of the phenoxy acids

Even though, no reports could be found regarding the degradation pathways of phenoxy acids under aquifer conditions to date, it could be speculated that they are similar to the degradation pathways reported from topsoil experiments. The metabolic pathway of phenoxy acids have been extensively studied in bacterial pure cultures. Especially, the pathway driven by the model organism *Cupriavidus pinatubonensis* (formerly *C. necator*) JMP134, which harbours a transmissible plasmid, pJP4, containing all the regulatory and structural genes encoding for the enzymatic degradation of 2,4-D and related compounds (Don et al., 1985; Pieper et al., 1988; Ledger et al., 2006; Perez- Pantoja et al., 2008). The entire pathway of MCPA in *C. pinatubonensis* JMP134 is given in Figure 4.1.

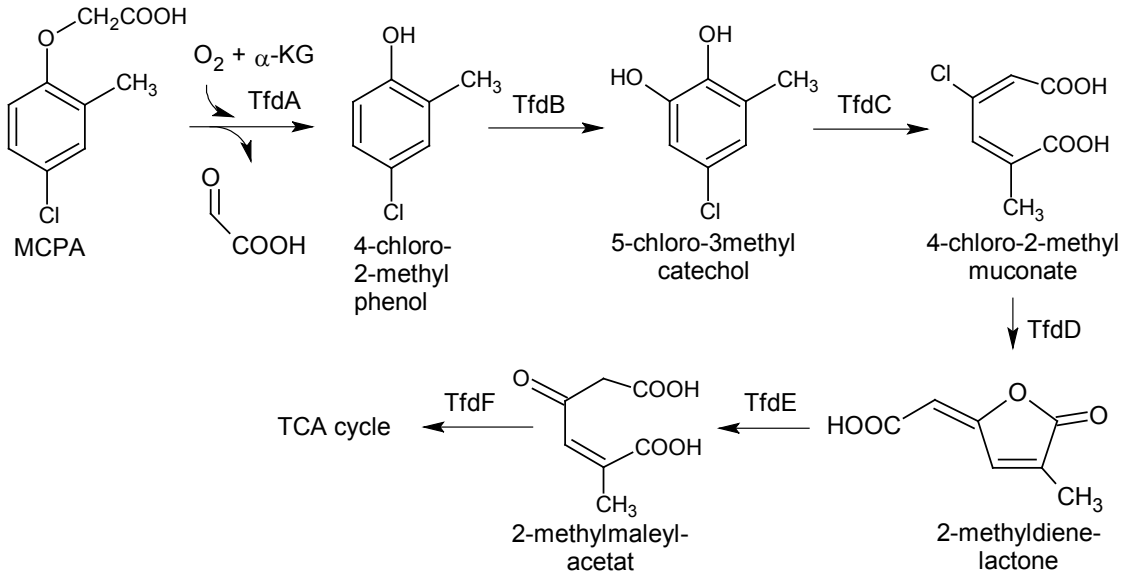

Figure 4.1. Degradation pathway of MCPA in *C. necator* JMP134. The enzymes involved in the degradation steps are shown above the arrows (modified from Roberts et al., 1998).

The first step in the degradation of MCPA is the cleavage of the ether linkage to produce 4-chloro-2-methlyphenol (MCP) (Fukumori and Hausinger, 1993a), initiated by oxygenases encoded by *cadAB* or *tfdA*-like genes (that are *tfdA* and *tfdAα*) (Streber et al., 1987; Itoh et al., 2002, 2004; Kitagawa et al., 2002). The remaining steps in the degradation pathway are encoded by *tfdB-F* genes (Don and Pemberton, 1985). Futher enzymatic reactions encoded by chromosal genes continue through the Krebs tricarboxylic acid cycle (Roberts et al., 1998) and

results in the production of CO_2, H_2O and chloride as the final products (Don et al., 1985).

4.4. Phenoxy acid degraders

Numerous strains capable of degrading phenoxy acids have been isolated from various different environments. Mainly, they have been isolated from agricultural soils and water bodies affected by several types of pesticides (Pemberton et al., 1979; Tonso et al., 1995; Vallaeys et al., 1998; Smejkal et al., 2001), as well as from pristine locations such as lava sealed soil from Hawaii and non-treated soil from Japan (Kamagata et., 1997; Itoh et al., 2000). These bacteria are categorized into three groups based on their evolutionary and physiological basis (McGowan et al., 1998; Itoh et al., 2002, 2004). Group I consists of *β*- and *γ*-subdivions of proteobacteria, which harbour *tfdA*-like genes, This group can be further divided into three classes (classes I to III) based on their *tfdA* gene sequences (McGowan et al., 1998) (see section 4.5). The second group includes the cluster of *α*-proteobacteria belonging to the *Bradyrhizobium*- related organisms, while group III consists of *α*-proteobacteria closely related to *Sphingomonas*-related organisms (Fulthorpe et al., 1995; Kamagata et al., 1997; Zaprasis et al., 2010). Group II organisms harbour both *tfdA*-like and *cadA* genes, whereas only *cadA* was detected in group III organisms (Itoh et al., 2002, 2004; Kitagawa et al., 2002; Huong et al., 2007, 2008) (Figure 4.2).

The majority of the strains capable of degrading phenoxy acids have been isolated by using 2,4-D as substrate. Naturally, most of the research involved on catabolic genes and classification of degrader strains has been done based on these strains. The similar compound MCPA has been less studied, and to my best knowledge, no bacterial degraders have been isolated based on this compound. It is a well known fact that the medium composition used in the cultivation process is likely to be the main determining factor for the selection of organisms with specific characteristics and catabolic pathways (Veldkamp, 1973; Harder and Dijkhuizen, 1982). Even though the first step in the degradation of MCPA and 2,4-D is the same, only few of the strains isolated on 2,4-D have been investigated for their ability to degrade MCPA (Smejkal et al., 2001) and the ones that degrade were considerably slower degrading MCPA than 2,4-D (Bælum et al., 2010). Furthermore, differences in functional diversity between the degrading populations were reported in studies on expression of the functional *tfdA* gene during degradation of these compounds *in situ* in soil (Bælum et al., 2006). Thus, isolation of MCPA degrading bacteria is an emerging

need for gaining better knowledge on the capability and actual activity of the microbial MCPA-degrading community in the environment.

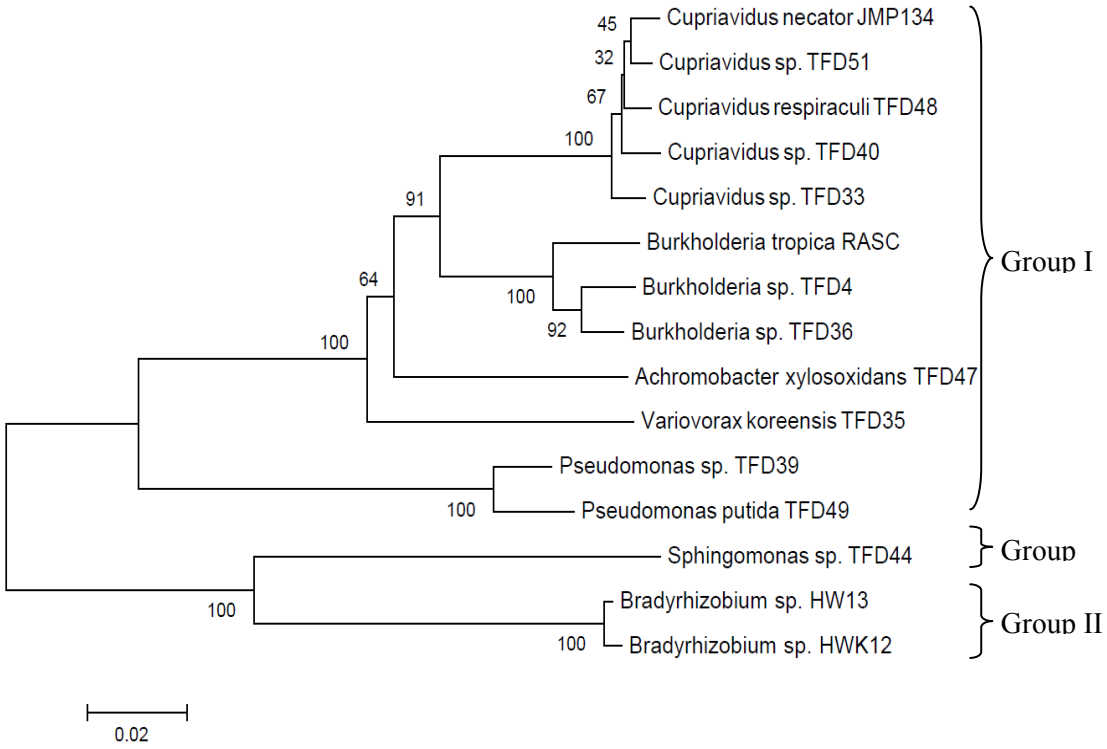

Figure 4.2. An extract of the phylogenetic relationship of 2,4-D degraders based on 16S rRNA genes. The phylogenetic tree was constructed based on ~ 1200 bp sequences using the Neighbour Joining method. Bootstrap values are shown at the nodes. The scale bar indicates substitutions per site (modified from Bælum et al., 2008a; Bælum et al., 2010).

In this study, five different MCPA degraders were isolated using a novel cultivation approach (**Paper II**). The 16S rRNA gene sequences obtained from the isolates, originated from the same sediment material, were diverse and included members of all three divisions of *Proteobacteria*. This broad diversity of the isolates, obtained from the same sediment material, is contradictory to the observations of Kamagata et al. (1997) who reported that 2,4-D isolates isolated from sites with historical exposure of typically belong to β and γ subdivisions of *Proteobacteria,* while bacteria from pristine environments belong to the group of α- proteobacteria. Table 4.2 presents the MCPA degrader pure bacterial strains obtained in this study.

Table 4.2. List of isolates obtained in this study. Closest cultivated type strains were determined by comparing 16S rRNA gene sequences to the ones deposited in the Genebank Database.

Species	Closest cultivated type strain		Functional genes		
Isolate	Type strain [accession no.]	% homology (bp)	*tfdA*	*tfdAα*	*cadA*
ERG1	*Achromobacter piechaudii* strain TZ4 [GQ927161.1]	99 (1389/1390)	X	X	X
ERG2	*Pseudomonas fluorescens* strain S16 [DQ095904.1]	99 (1410/1411)	X		X
ERG3	*Variovorax paradoxus* strain B57 [EU169160.1]	99 (1401/1420)	X		X
ERG4	*Cupriavidus basilensis* strain AU4546 [AY860224.1]	98 (1385/1408)	X		X
ERG5	*Sphingomonas sanxanigenens* strain T12AR21 [JF459935.1]	97 (1319/1356)	X		X

4.5. The diversity of functional genes

As described in section 4.3, the first step of the phenoxy acid degradation is catalyzed by an oxygenase, which is mostly encoded by the *tfdA* genes. It has been shown that diversity among the *tfdA* genes is high (Vallaeys et al., 1996; Kamagata et al., 1997). McGowan et al. (1998) suggested classifying *tfdA* genes into three classes (I–III) with sequences between classes showing 75–80% homology. Class I *tfdA* genes consists of those of *Cupriavidus pinatubonensis* JMP134, while class II *tfdA* genes are closely releated to those of *Burkholderia* sp. strain RASC (McGowan et al., 1998). Recently, Bælum et al. (2010) suggested portioning *tfdA* Class I into two subclasses (classes I-a and I-b) based on differences in the nucleic acid sequences. Class III *tfdA* genes are 77% identical to class I and 80% identical to class II *tfdA* genes. Class I and III *tfdA* genes were obtained from bacteria belonging to many different families, while class II genes were found only in the *Burkholderia* branch (Bælum et al., 2010). Apart from the *tfdA* genes, *tfdAα* and *cadA* genes, which also encode the first enzymatic conversion of phenoxy acids, were isolated and characterized (Itoh et al., 2002; Kitagawa et al., 2002). These genes have their own respective groups and there seemed to be pronounced diversity within the *cadA* genes (Itoh et al., 2004). However no classification of *cadA* genes into classes have been proposed yet.

Bælum et al. (2006) suggested that class III *tfdA* harbouring organisms were responsible for the majority of MCPA degradation in soil, and the organisms possessing class I *tfdA* genes were unable to achieve all the steps of MCPA

degradation. The lack of correlation between the different classes of *tfdA* genes might reflect the difference between the natural soils studied and the selective nature of analyzing bacterial isolates. Besides, Bælum et al. (2010) stated that a possible explanation for this phemenon may be that the the strains they used were biased by their substrate of isolation being 2,4-D. This hypothesis has been supported by our findings **(Paper II)**. Along the isolated MCPA degraders originated from the same sediment material that had a history of MCPA exposure, the *tfdA* sequence of the isolates were similar to the *tfdA* gene either from *Cupriavidus necator* JMP which harbours class I-a genes *tfdA* genes, or from *Achromobacter xylosoxidans* subsp. *denitrificans* EST4002 which is classified as *tfdA* class I-b gene (Bælum et al. 2010). In contrast to the general acceptance in the literature, we have shown that *tfdA* class I gene harbouring bacteria is involved in MCPA mineralization in samples that were subjected to MCPA exposure (**Paper II**). Furthermore, all the MCPA degrading isolates obtained in this study harboured *cadA* genes. This is in parallel with the recent findings of Liu et al. (2010) who showed *cadA*-hosting microorganisms were enriched during MCPA degradation, indicating that such organisms are involved in MCPA degradation. In addition to *tfdA* and *tfdA*-like genes, the *rdpA* and *sdpA* genes, showing 50% identity to *tfdA* genes, have been shown to be involved in the degradation of some phenoxy acid herbicides (Westendorf et al., 2002; Paulin et al., 2010). However these genes are more specific to phenoxy acid herbicides MCPP and dichloroprop, which is not in the scope of this thesis, therefore will not be discussed.

Several authors reported that *tfdA* and *cadA* genes are mobile and potentially readily distributed by horizontal gene transfer based on the by phylogenetic comparison of these genes and 16S rDNA genes (Ka et al., 1994; Fulthorpe et al., 1995; de Lipthay et al., 2001; Zakaria et al., 2007). These findings were supported also in this thesis. The incongruity of the *tfdA* and *cadA* genes with the 16S rRNA genes along the obtained isolates could suggest that horizontal transfer of these genes occurred either in the aquifer, or during the enrichment process (**Paper II**).

5. Conclusions and perspectives

In this PhD study, including the following manuscripts, our understanding of degradation of low pollutant concentrations in oligotrophic environments has been deepened. Novel approaches for cultivating pesticide degrading bacteria that are specially adapted to low substrate concentrations were presented. Furthermore, the mechanisms and bacterial physiology underlying the occurrence of low contaminant concentrations were studied.

It was hypothesized that classical enrichment cultures using rather high pollutant concentrations may have overlooked bacteria that are able to metabolize substrates at low concentrations and are thus possibly more efficient at remediating low levels of organic pollutants. In order to test the hypothesis, we have investigated the differences in metabolic activity, community structure and dynamics, population growth, and single cell physiology of two MCPA degrading enrichment cultures obtained by a conventional enrichment approach based on low (100 μg L^{-1}) or high (25 mg L^{-1}) MCPA concentrations originated from the same aquifer material. We have demonstrated that different populations of herbicide-degrading bacteria adapted to metabolize substrates at different concentrations can exist in a community. We have also shown that using environmentally relevant substrate concentrations, in contrast to the classical high concentrations, provide bacterial communities that are more efficient in terms of metabolic functionality and stability. Furthermore, the presence of low nucleic acid (LNA)-content bacteria was positively correlated with mineralization activity in cultures enriched on low MCPA concentration, but not in cultures enriched with high levels of MCPA. This suggests that LNA bacteria are active populations which could play a role in degradation of low pollutant concentrations and they might be overlooked in cultivation studies which typically use high levels of substrate. Even though it has been reported that LNA-bacteria are better adapted to low substrate environments, this was the first report showing a direct link between the growth of LNA bacteria and the metabolic activity occurring at low substrate concentrations.

The proliferation of LNA bacteria in communities selected with low MCPA concentrations bring out further questions that were tried to be answered in this study. We hypothesized that the MCPA degrader strains that were isolated by low concentrations and that are more efficient at degrading low levels of MCPA would be dominated by LNA cells and increasing the substrate concentration would have a negative effect on LNA populations. In consistence with our

hypothesis, we have shown that the strains isolated at low levels of MCPA were dominated by LNA cells and this dominancy decreased with increasing MCPA concentrations. However, the strains that were cultivated with high MCPA concentrations showed strict HNA properties regardless of MCPA concentration. These results are also valuable to understand the bimodal distribution and interactions between these groups, which are not yet to be fully explained. We suggest that the occurrence and distribution of these two fractions are the result of complex processes that includes both dynamic exchanges between the LNA and HNA cells as well as characteristics that are distinct to each of the fractions. I believe that understanding the role of LNA bacteria and their characteristics is a key factor for isolating such bacteria that are possible candidates for bioremediation of environments contaminated with low concentrations.

One of the major aims of this thesis was to develop novel cultivation techniques for isolating bacteria from oligotrophic aquifers that are efficient at degrading low pollutant concentrations. In this perspective we have developed a plate-based isolation method, called Low Flux Filter (LFF) plates, which relies on providing an inoculum with low diffusive fluxes of substrate for extended incubation time. The method was successfully applied to isolate MCPA degrading bacteria either directly from groundwater sediment extracts or enrichment communities obtained at low (100 μg L^{-1}) or high (25 mg L^{-1}) MCPA concentrations. The isolated strains, the first MCPA-degraders isolated directly on MCPA, exhibited different degradation kinetics when tested with different MCPA concentrations. It has been concluded that the bacterial strains isolated from the cultures enriched with low MCPA concentrations were more efficient in mineralizing MCPA at low concentrations (1 μg L^{-1}) than the ones isolated from the cultures enriched with high levels of MCPA. The functional genes that catalyze the first step (*tfdA*, *tfdAα* and *cadA*) showed differences between the strains in regards to the isolation source where they were obtained. The *tfdAα* gene was only present in the strain that were isolated from sediment extract. The *tfdA* and *cadA* genes were similar in the strains that were isolated from the enrichment cultures, whereas they were different in the strain isolated from the sediment extract. This suggests that horizontal gene transfer plays dominant role for some strains to acquire these genes during the enrichment process.

Bioaugmentation, i.e the introduction of microorganisms with specific catabolic capabilities into a contaminated environment, is an environmentally friendly and useful method for the removal of micropollutants. This technology can be used in treatment systems such as sand filters, activated carbon filters, sand barriers or

mobile biofilters, as well as in contaminated sites and aquifers. However, bioaugmentation is often unsuccessful due to several reasons such as predation by protozoa, abiotic stress, competition with the indigenous microbes and low concentrations of pollutants in the environment. Detailed knowledge of activity, diversity and physiology of bacteria specialised at degrading low contaminant concentrations is limited. And, most of the strains obtained in our laboratories are biased due to their selection on high contaminant concentrations. The initial strain selection step is a key aspect for successful bioaugmentation approaches. Thus, selection of microorganisms specially adapted to metabolize low concentrations of pollutants and identifying their characteristics is an emerging need. In this study we have shown that aquifers harbour microbial populations that are specifically adapted to low pesticide concentrations, whose potential can be accessed using specific cultivation approaches. Some of the characteristics of these populations at community and individual level were identified by the use state-of-the-art methods. The results of this thesis would contribute significantly to our understanding in order to overcome some of the limitations explained above and to develop efficient bioremediation technologies for the removal of organic contaminants in groundwater and surface water at low concentrations.

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7. Papers

I. Gözdereliler, E., Boon, N., Aamand, J., De Roy, K., Granitsiotis, MS., Albrechtsen, HJ., Sørensen, SR. Comparing metabolic functionality, community structure and dynamics of herbicide-degrading communities cultivated with different substrate concentrations, submitted manuscript.

II. Gözdereliler, E., Dechesne A., Nicolaisen, MH., Sharp, RE., Aamand, J., Albrechtsen, HJ., Smets, B., Sørensen, SR. Isolation of 2-methyl-4-chlorophenoxyacetic acid degrading bacteria from groundwater sediments using a novel low substrate flux approach, submitted manuscript.

III. Gözdereliler E, De Roy K, Boon N, Sørensen SR. Substrate concentration alter the cytometric characteristics of herbicide degrading bacteria with high and low nucleic acid content, submitted manuscript.

The papers are not included in this www-version, but can be obtained from the Library at DTU Environment. Contact library@env.dtu.dk or Department of Environmental Engineering, Technical University of Denmark, Miljoevej, Building 113, DK-2000 Kgs. Lyngby, Denmark.

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