The Microbiology of Railway Tracks

Towards a Rational Use of Herbicides on Swedish Railways

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Abstract

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Swedish railways are regularly treated with herbicides in order to keep the track beds free from weeds. However, finding appropriate preparations and dosages that provide a good weed control but that are still environmentally acceptable, has proven to be difficult.

This thesis investigates some fundamental aspects of the microbiology of railways, such as amounts, activities and spatial distributions of microorganisms in the track bed, in order to provide the knowledge base for a more informed use of herbicides on railway tracks.

The railways investigated were characterized by overall low but highly variable rates of respiratory activity. Distributions were positively skewed and autocorrelation distances were short. Microbial biomass measured as substrate-induced respiration (SIR), microbial activity measured as basal respiration and a kinetically derived parameter *r* corresponding to the active fraction of the SIR covaried significantly with the organic matter content of the ballast. For basal respiration and *r*, the most important covariate was the water content of the ballast and microbial activity was higher when determined in the autumn during moist conditions. The functional diversity, assessed as substrate richness on Biolog ECO plates, was low but highly variable and covaried with SIR, indicating that functional groups of microorganisms were missing where the microbial biomass was low. The substrate utilization patterns were homogeneous among the railway samples, which suggest that heterotrophic microorganisms are randomly distributed on railway tracks.

Degradation of diuron in fine material of railway ballast followed first-order kinetics and thus did not support growth of degrading microorganisms. The metabolites DCPMU and DCPU were formed in all samples and accumulated in most of them. The mineralization of MCPA followed growth-linked degradation kinetics and was enhanced where the railway track had been previously treated with MCPA. This enhancement was related to higher numbers of MCPA-degraders and higher specific growth rates (μ) of these in the previously treated track. The yield (Y) correlated to the nitrogen content of the railway, indicating that the formation of microbial cells from MCPA on railways is nutrient limited.

These findings indicate that it would be sensible to use metabolically degradable herbicides and to apply them using weed-seeker techniques in order to decrease the likelihood of groundwater beneath the track being contaminated.

Keywords: railway tracks, substrate-induced respiration (SIR), functional diversity, diuron, MCPA, metabolic and cometabolic degradation

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I den unga sjudande huvudstaden,
vid det nya stolta riksdagshuset där,
alldeles nedanför gaveln,
under republikens hjärta,
där stod jag och såg på det kvarglömda spåret,
en skärning med rostiga skenor,
ogräs och grus mellan ruttna sleepers
– hörde på dem som var med mig:
(urskuldande)
Snart skall här fyllas igen!

Så ge mig minnet av grus och ogräs,
malört, kardborre, tistel,
och spår som ingenstans ledde.
Ge mig det värdelösa,
det som har tjänat ut
och kan återgå till sitt ursprung,
det som har tjänat nog
att adlas av glömska och vanvård!
Lyckliga ting som får vara sig själva
vittra och rosta ifred!
Jag känner för dem!

Gunnar Ekelöf

Abbreviations/terms/common names

AOB	ammonia oxidizing bacteria
AWCD	average well-colour development
CLPP	community-level physiological profile
CTC	5-cyano-2,3-ditolyltetrazolium chloride
2,4-D	2,4-dichlorophenoxyacetic acid
DCA	3,4-dichloroaniline
DCPU	1-(3,4-dichlorophenyl) urea
DCPMU	1-(3,4-dichlorophenyl)-3-methyl urea
DGGE	denaturing gradient gel electrophoresis
DNOC	4,6-dinitro-*ortho*-cresol
Diuron	3-(3,4-dichlorophenyl)-1,1-dimethyl urea
Glyphosate	*N*-(phosphonomethyl)glycine
Imazapyr	(*RS*)-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid
K	'dormant' non-growing fraction of the SIR response
MCPA	4-chloro-2-methylphenoxyacetic acid
qCO_2	metabolic quotient; ratio of basal respiration to microbial biomass
r	'active' growing fraction of the SIR response
SIR	substrate-induced respiration
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
TCDD	2,3,7,8-tetrachlorodibenzo-*para*-dioxin
TRFLP	terminal restriction fragment length polymorphism
VBNC	viable but non-culturable

Contents

Appendix

Papers I-IV

This thesis is based on the following publications, which are referred to by their Roman numerals:

I. Cederlund, H. and Stenström, J. 2004. Microbial biomass and activity on railway track and embankments. *Pest Management Science* 60: 550-555.

II. Cederlund, H., Thierfelder, T. and Stenström, J. 2006. Low microbial biomass, activity and functional microbial diversity on Swedish railways. (Submitted).

III. Cederlund, H., Börjesson, E., Önneby, K. and Stenström, J. 2006. Metabolic and cometabolic degradation of herbicides in fine material of railway ballast. (Submitted).

IV. Enwall, K., Nyberg, K., Bertilsson, S., Cederlund, H., Stenström, J. and Hallin, S. 2006. Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. (In revision with Soil Biology and Biochemistry).

Paper I is reprinted with permission of the publisher.

My contributions to the papers were:

I. Planned the work and conducted the field sampling together with Dr Stenström. Performed all the laboratory work and data analysis. Had the main responsibility for writing the manuscript.

II. Planned the work together with my co-authors and performed the field sampling together with Dr Stenström. Performed all the laboratory work. Analysed the results together with my co-authors. Wrote most of the manuscript excluding the statistics section.

III. Planned the work together with Dr Stenström and performed the field sampling. Performed most of the laboratory work, excluding the analysis of diuron and MCPA by HPLC and GC. Wrote the major part of the manuscript excluding the material and methods sections concerning the HPLC and GC-analyses.

IV. Helped with the analysis of the respirometric data and wrote parts of the material and methods, results and discussion sections dealing with these.

1 Introduction

On the 5th of March 1856, almost exactly 150 years ago, a combined passenger and freight train steamed between Örebro and Nora, representing the inauguration of the first public Swedish railway. Since then, the Swedish railway net has vastly expanded, and today it encompasses almost 12,000 km of track, stretching from Trelleborg in the south to Riksgränsen in the north. For safety purposes these tracks need to be maintained in good condition and one important aspect of this work is to limit weed infestation and growth. For this purpose, chemical weed control has been, and still is, primarily used (Torstensson, 2001).

Using herbicides on railway tracks is neither uncomplicated nor uncontroversial. A number of Swedish railway workers who handled herbicides in the late 1950s and early 1960s developed cancers and died prematurely (Axelson *et al.*, 1980) and the memory of this to some extent still affects the attitude of the Swedish public towards herbicide-use on railways. Later weed control efforts caused the unintentional killing of pine trees along certain railway stretches (Torstensson *et al.*, 2002) and several studies have indicated that herbicides are very persistent in railway embankments, and that they may leach and contaminate groundwater (Börjesson *et al.*, 2004; Lode & Meyer, 1999; Ramwell *et al.*, 2004; Schweinsberg *et al.*, 1999; Torstensson, 1994; Torstensson *et al.*, 2005).

Today, as rail travel and conveyance of goods by rail are being promoted as a more environmentally sound alternatives to fossil fuel-driven transport, it is increasingly important for the Swedish Railway Administration (Banverket) to be able to convincingly demonstrate to both the Swedish Chemicals Inspectorate and to the Swedish public that the herbicides currently being used have no adverse effects. Consequently, since the end of the 1980s, Banverket has funded a research programme at the Department of Microbiology, Swedish University of Agricultural Sciences (SLU). This programme is aimed at assessing not only the efficiency of herbicides for weed control on railways but also how they are degraded and adsorbed in railway embankments (Torstensson, 2001). It was within this contextual framework that the present thesis on the microbiology of railways was conceived and the exploratory work was carried out.

This thesis aims to answer some fundamental questions about the amounts, activities and distributions of microorganisms on railway tracks, all of which are crucial to obtaining a more comprehensive view of the fate of herbicides applied on railways and a more complete understanding of the potential for herbicide dissipation in the railway environment. These research findings are placed in perspective with an account of historical herbicide use on Swedish railways and a contribution (the railway perspective) to the largely untold story of the Swedish 'Hormoslyr debate' of the 1970s.

Måste den som åker tåg
tänka rälstankar?
Werner Aspenström

2 The railway track

Railway tracks are designed to withstand the strains and stresses that arise from the operation of trains at high speed or with heavy loads, as well as all kinds of environmental wears. The rails are supported by the ties (sleepers), which are supported in turn by the track bed (Figure 1). The ties should be distributed with a spacing of 0.65-0.5 m depending on the types of loads that are expected on the track. The function of the ballast is to stabilize the construction in all directions, distribute the weight between ties and subgrade, render elasticity to the track and provide good drainage. Therefore, the rule is that the ballast should comprise a 30-60 cm layer of coarse crushed stones, so called macadam, named after the Scottish engineer John Loudon McAdam. The subgrade (Figure 1) should preferentially be made out of gravel and/or coarse sand. Its functions are to distribute weight, drain the railway embankment and protect the ballast from so called ballast pumping, a process whereby fine materials is being pushed up into the ballast because of the heavy load (Sundquist, 2003).

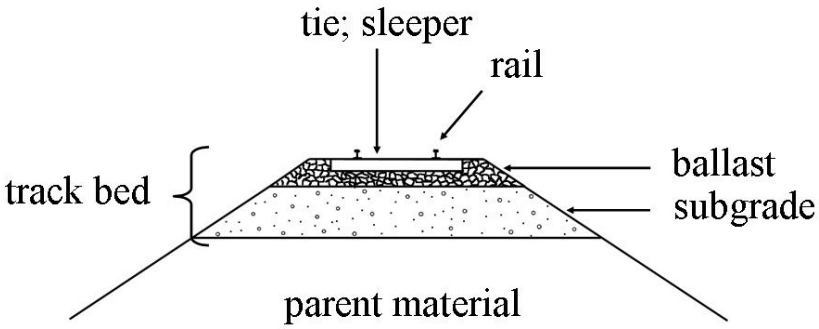

Figure 1. Cross-section of a railway embankment (after Sundquist, 2003).

However, railway type described above represents somewhat of an ideal case from a constructional point of view. Newly constructed tracks can look like this but there are also many older types of tracks in Sweden that are constructed primarily from gravel, sand or till. The macadam layer may be very thin, contaminated with finer materials or sometimes not present at all. In these kinds of embankments, there is not always a clear distinction between ballast, subgrade and parent materials, wooden ties are irregularly distributed and the track bed is often infested with weeds.

3 Weed control on railway tracks

3.1 The need for weed control on railways

There are several reasons why weeds need to be removed from railway tracks. Tall weeds can reduce visibility for engine drivers, who need to have a clear view of signals and railway crossings. Furthermore, weeds may obscure rails and switches, making inspections of the railway line more difficult (Anonymous, 2005b). Wet fallen leaves during autumn, or weeds that are mashed over the rails can lead to reduced friction between the wheels and the rails so that trains slip or skid when trying to accelerate or, even more importantly, when trying to come to a halt (Berggren *et al.*, 2004; Torstensson, 2001). Decaying plants that contaminate the macadam or gravel ballast can cause drainage problems by clogging pores and binding water. This can lead to a reduced resilience of the embankment, which could render the tracks unsuitable for the operation of high speed trains (Müller, 2001). Compromised structural integrity and impaired drainage function of the ballast can in extremes lead to derailment (Anonymous, 2005a). Furthermore, water in the track bed may freeze in the winter and cause track dislocations, and moisture also reduces the life length of wooden sleepers, which are still used on many Swedish railway stretches (Torstensson, 2001). Thus, weed control on railways is important for security reasons. However, as can be deduced from the following sections, an important lesson that can be drawn from the 20th century use of herbicides is that it is also important to make sure that the methods used for weed control are safe themselves, both for the people that carry out the work and for the surrounding environment.

3.2 A short history of weed control

3.2.1 Non-chemical weed control

The need for good weed control in agriculture was well understood already by the classical writers of the Greek and Roman civilisations. Early efforts in weed control are likely to have been of a mechanical nature such as hand weeding (mentioned by Theophrastus, 4th century B.C.) and ploughing (mentioned by Xenophon, 4th century B.C.) as well as weeding with hoes (Virgil 70-19 B.C.) and sickles (Columella 1st century A.D.) (Smith & Secoy, 1975). An early critic of weed control was apparently Jesus, who in the parable of the weeds, likens the weeds of the field to the sons of the evil one, but still advises against weeding since: ‘while you are pulling the weeds, you may root up the wheat with them’ (Matthew 13:29). In the Geoponika, a compilation of agricultural writings that were assembled from earlier compilations of Roman and Greek writers and that was published in the 10th century A.D., readers are recommended to dig round ground sown by human hand or to harrow it by means of oxen. Weeding with instruments once the crop begins to cover the grounds is also recommended, and if the grounds is weeded twice the utility is said to be doubled (Leontinus, 1805). In a more recent example of weed control literature, the Swedish scientist Pehr Adrian Gadd lists a number of measures for preventing weed spread and germination, as well as harrowing and ploughing as sound practices for removing

weeds from agricultural lands (Gadd, 1777). Still at the beginning of the 20th century mechanical and manual methods were the most important (Bohlin, 1903; Clark & Fletcher, 1909). However, the development of mechanical devices into more advanced horse-drawn cultivators, weeders and hoes continued throughout the 18th to 20th centuries, and in the 1940s new innovations such as flame cultivators and electrovators came into some use (Timmons, 2005). Many advances in non-chemical weed control have been made in recent decades, including thermal measures such as the use of hot water (Hansson, 2002), infrared light (Ascard, 1998), CO_2 lasers (Heisel *et al.*, 2001) and microwaves (Sartorato *et al.*, 2006).

Several of the classical works on agriculture make little distinction between advice that is of a practical nature and advice that is of a more religious or magical nature, and sometimes such advice can be quite amusing. For example, according to the Geoponika, clearing the land completely of a weed called lion's tail can be achieved by placing shells with drawings in chalk of Hercules suffocating a lion in the corners and at the centre of the field. Letting someone, possibly 'a marriageable virgin, having her body and feet naked, without any the least clothing, with dishevelled hair', go round the field carrying a cock, that, while it was in a state of consternation, has been looked at attentively by a lion, is also suggested to intimidate the weed (Sotion, 1805)! Irrespective of how tempting these practices may have seem to its readers, the simple but labour-intensive practices of hand weeding, harrowing and ploughing have continued to be the most used methods for removing weeds from agricultural fields until the rapid development of herbicides started in the mid 20th century.

3.2.2 Chemical weed control

Herbicides, as we know them today, were not used by the ancient Greek and Roman civilizations but there are some early references to practices that can be taken as examples of chemical weed control. According to the 'Natural History' (XVIII:8), written by Pliny the Elder (23-79 A.D.), Democritus (5th century B.C.) proposed that lupin flowers soaked in hemlock juice could be used to clear forests (Pliny, 1855). Theophrastus wrote that trees could be killed by pouring oil on their roots and the Roman writers Cato (234-149 B.C.) and Varro (116-17 B.C.) recommended the use of amurca, a waste product from olive oil production, for weed control. Varro mentions specifically that amurca was poured around olive tree roots and 'wherever noxious weeds grow in the fields' (Smith & Secoy, 1975).

The phytotoxic properties of common table salt (sodium chloride) were well known already in ancient times and when the Romans annihilated Carthage they ploughed salt into the fields in order to ensure that nothing could grow there (Smith & Secoy, 1975). Indeed, modern research and development of herbicides were initially directed towards the use of different inorganic salts and acids, and many of these early herbicides were so-called soil sterilants or sterilizers that rendered the soil unfit for growing plants until they had been washed out by rain (Hildebrand, 1946). Early trials with the use of chemicals were carried out in

Germany during the 19th century and included lime, sodium chloride, chlorcalcium, sulphuric acid and iron sulphate (Mukula & Ruuttunen, 1969). The development of selective weed control started in 1886 in France, where Bonnet, who had noted the phytotoxic effects of copper salts, sprayed copper sulphate solution on a field with oats and managed to selectively kill wild radish and charlock while leaving the crop unharmed. In the early 20th century, many chemicals, including cupper sulphate, iron sulphate, ferrous sulphate, ammonium sulphate, sodium arsenate, sodium arsenite, as well as sulphuric and nitric acids were tested for their herbicidal properties by researchers in France, Germany and USA (Mukula & Ruuttunen, 1969). Progress in weed control prior to World War II was rather slow but included the discovery by French scientists that the yellow dye dinitro-*ortho*-cresol (DNOC) could be used for selective weed control (Blackman, 1948). Also Petroleum herbicides, such as undiluted dry-cleaning fluids, gasoline, diesel and kerosene also came into use prior to 1950, especially on non-crop areas and for the selective weeding of carrots (Blackman, 1948; Hildebrand, 1946; Osvald, 1947; Timmons, 2005).

Early Swedish research on herbicides was carried out by Sigurd Rhodin, who tested 15% ferrous sulphate solution in field trials already in 1898 (Rhodin, 1903). Later trials in 1909-1910 also included calcium cyanamide, which proved to be almost equally effective for selective weed control (Rhodin, 1911). However, a survey of Swedish farmers in 1921 revealed that the use of chemicals was still not very widespread, although a few of the farmers stated that they used ferrous sulphate (Adolfsson, 1995). Osvald (1947), in his report on weed research carried out at the Institute of Plant Husbandry, Royal Agricultural College of Sweden, in the years 1935-1947, lists the susceptibility of weeds and crops to sodium chlorate, calcium cyanamide, sulphuric acid, copper sulphate, DNOC and the recently discovered hormone derivatives (without specifying which of these that were used in the trials). Out of these, those most commonly in practical use in Sweden at the time appear to have been sulphuric acid and the copper sulphate.

Several of the early 20th century herbicides, such as sodium arsenate (Hildebrand, 1946) and DNOC (Osvald, 1947), were known be toxic to livestock, fire-hazardous and explosive, and the selectivity of many of the chemicals that were used for 'selective weed-control' was not all that good. Considering this, one can appreciate how overwhelmingly positive the new weed killers, the hormone herbicides 2,4-D, 2,4,5-T and MCPA, were perceived, and how they revolutionized agriculture upon their introduction in the mid 1940s (Troyer, 2001). After World War II, the overall use of herbicides in agriculture, as well as the development of new herbicides sky-rocketed (Appelby, 2005; Timmons, 2005; Troyer, 2001) and many of these new compounds also found their ways to the railways.

3.3 Historical and current use of herbicides on Swedish railways

3.3.1 Weed control on Swedish railways prior to 1971

Weed control on Swedish railways was initially carried out manually by so-called lengthmen. These were railway workers who were employed by the state railways (SJ; currently a railway transport operator, prior to 1988 also responsible for constructing and managing Swedish railways) and who lived in special lengthmen's cottages that were distributed along the tracks, usually about 2.5-4 km apart. It was the responsibility of the lengthmen to inspect and to maintain their sections of track and this included the removal of weeds (Lindmark, 1991). The first chemical that was used for weed control on railways was sodium chlorate, in a formulation called Klorex 55, from 1925 until 1957 (Skoog, 2006). Klorex had some obvious drawbacks in that it was very fire hazardous, potentially explosive, corrosive and electrically conductive (Torstensson, 2006). Furthermore, Klorex was a contact herbicide that had no or very little residual effect on weeds (Beinhauer, 1962). Consequently, in the mid 1950s, research was carried out in cooperation with the Royal Agricultural College in order to find more effective and less dangerous herbicides for weed and brush control (Beinhauer, 1962). Klorex was replaced by an array of different herbicides that were used from 1957 and on throughout the 1960s (Tables 1 and 2). The active ingredients that were used in the largest quantities during this period were amitrole for weed control and 2,4,5-T and 2,4-D for brush control.

3.3.2 Environmental concerns and the Hormoslyr debate

In 1971, it was reported that some of the personnel who had handled the herbicides in the 1950s and 1960s had retracted cancer, and SJ decided to temporarily stop all herbicide applications (Skoog, 2006). As witnessed by Eric Jönsson in the book *Dödens tåg* (Eng. 'The Train of Death'), in the first years after the introduction of the chemicals that came into use in the 1950s, everybody who worked with chemical weed control was convinced that the new herbicides were completely harmless. Therefore, the workers that handled the spraying equipment did not wear protective clothing and the working environment was such that they were continuously exposed to the herbicides. The spraying train even had the spraying nozzles mounted in front so that the engine driver, who had to lean out of his window to see where he was going was often inhaling a mist of chemicals (Johansson & Jönsson, 1991). Eventually, several of the railway workers who had handled or come into contact with the herbicides started to display various symptoms of illness such as aches, tumours and vascular spasms, and many died prematurely. At first, these symptoms were not linked to their exposure to herbicides but as chemical awareness grew in the 1960s, so did the suspicion that these compounds might be responsible.

Table.1 Chemicals used for weed control on Swedish railways

Name of formulation	Active ingredients	Years of usage[1]
Klorex 55	sodium chlorate	1925-1957
Totex	atrazine + dichlobenil[2]	1957-1958
Ureabor	disodiumtetraborate + monuron	1958-1960
Emisol 100/Emisol 50	amitrole	1958-1971
Telvar	monuron	1959-1962
Primatol A	atrazine	1959-1962
Weedex tel/kar	amitrole + diuron	1961-1971
Primatol D43	atrazine + mecoprop + 2,4,5-T	1963
Hyvar x	bromacil	1963
Totalex Extra[3]	atrazine + dichlorprop + 2,4-D + 2,3,6-TBA	1968-1970
Uridal	diuron + dichlorprop	1969-1970
Totex strö[4]	atrazine + dichlobenil	until 1976
Karmex 80	diuron	1973[5]-1990
Karmex 80 df	diuron	1990-1992
Roundup	glyphosate	1986-87
Spectra	glyphosate	1988-1993
Roundup Bio	glyphosate	1993-present
Arsenal 250	imazapyr	1994-2002

[1] According to Skoog (2006) and Torstensson (2006).
[2] Probable ingredients.
[3] Was tested in the first year under the name Preparat C.
[4] Was mainly used for weed control around sheds, poles and signals.
[5] Only some limited testing of Karmex 80 was carried out in 1973.
The herbicide Gesaprim 50 slampulver (containing atrazine) was approved for use on railways by the Chemicals Inspectorate and it is possible that it was used at some time prior to 1970, although there is no record of this (Torstensson, 2006).

Table 2. Chemicals used for brush control on Swedish railways

Name of formulation	Active ingredients	Years of usage[1]
Brush killer/Brush killer 165	2,4-D + 2,4,5-T	1957; 61-64; 67
Esteron	2,4,5-T	1958; 67
Hormoslyr 64	2,4-D + 2,4,5-T	1959-61; 64-65; 69
Regulan	2,4,5-T	1964
Hormoslyr 500T	2,4,5-T	1964; 69
Triosid	2,4,5-T	1965
Herbexal 500D+T/500T	2,4,5-T	1968; 70
Mota Asp	2,4-D + picloram	1969

[1] According to Skoog (2006)

Although initially mainly amitrole was thought to be the agent involved in railway work related cancer (Axelson *et al.*, 1974), the public debate during the 1970s mainly revolved around the use of the formulation Hormoslyr. It had active ingredients in common with the herbicide formulation Agent Orange, which was used by the Americans for defoliation in large spraying operations during the

Vietnam war (Young *et al.*, 1978). These activities were very negatively perceived by the Swedish public and probably contributed to the increasing unpopularity of Hormoslyr, which was similarly used in Swedish forestry for selective control of broadleaved brush in coniferous plantations, both applied by hand sprayers and by airplanes (Jensen, 2006). It had also been disclosed that one of the ingredients of Hormoslyr, 2,4,5-T, was contaminated by the very potent toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and this was a cause of concern to the experts as well as to the public (Firestone, 1978). In the summer of 1970, the so-called Hormoslyr debate began in Sweden, in which the issue of Hormoslyr and whether it should be allowed in Swedish forestry gradually became a symbol for the struggle of the Swedish green movement (Bäckström, 1978; Enander, 2003). The debate was sometimes very intense, and the positions were locked. While the critics claimed that Hormoslyr was both carcinogenic and teratogenic and caused the deaths of reindeer, elk and forest users such as orienteers, as well as disease among berry-pickers, several of its advocates actually drank it, some of them in live broadcasts on Swedish television, in order to prove its harmlessness. Environmental groups destroyed airplanes and occupied clear cuts and on one occasion protesters were themselves sprayed with Hormoslyr from the air (Jensen, 2006). The use of phenoxy acids in forestry was temporarily banned in 1971 by the Poisons and Pesticides Board (which later became the Environmental Protection Agency), was allowed again in February of 1972 but was then again forbidden by law by the Parliament in March of the same year. This law was repealed in 1975 but in 1977 the use of 2,4,5-T, and thereby Hormoslyr, was completely forbidden (Bäckström, 1978). The protests from the environmental movement, however, continued until the use of herbicides eventually ceased completely in Swedish forestry (Enander, 2003).

In 1980, some railway workers who had handled the herbicides went to court in order to try to classify their ill-health as work related. However, they lost the case because more than 10 years had passed since they were exposed, and since this is the period of limitation for this kind of claim in Sweden (Johansson & Jönsson, 1991). Concerning the scientific basis for these claims, the unusually high tumour rate among railway workers who had been exposed to herbicides was documented by Axelson *et al.* in 1974 and 1980. However, no firm causal relationship to any specific compound could be established from their work – rather it appeared as if it was mainly workers who had been exposed to a combination of amitrole and phenoxyacetic acids who were affected (Axelson *et al.*, 1980). Another conclusion from their study was that the increased tumour incidence and shortened life expectancy was only seen among railway workers who had been exposed during the years 1957-61 and not among later exposures. This could indicate that the handling of the herbicides had gradually improved since then, or that the levels of contaminants during the production of 2,4,5-T had gradually decreased over time (Axelson *et al.*, 1980). Hardell & Sandström (1979) recorded a six-fold increase in the risk of soft-tissue sarcomas for people who had been occupationally exposed to phenoxyacetic acids (especially 2,4-D and 2,4,5-T) but could not establish whether the carcinogenic effect was due to these compounds or to impurities such as dioxins. These findings were initially very controversial, and have been criticized on methodological grounds, but today it is considered that the

accumulated scientific evidence has established that exposure to a combination of 2,4-D, 2,4,5-T and its associated contaminant TCDD can be linked to an increased risk of at least some forms of cancer (Hertz-Picciotto *et al.*, 2003). However, many, if not most of the claims that were made by the media and the environmental movement during the 1970s, concerning the effects of Hormoslyr and the phenoxyacetic acids were never substantiated (Bäckström, 1978; Enander, 2003; Ericson *et al.*, 1977; Newton & Young, 2004).

As previously mentioned, the use of the suspected carcinogenic herbicides Emisol and Hormoslyr on railways ceased already in 1971, when the first cancer incidents were reported. However, the opposition to the use of herbicides on railways and the attention that this issue was given in the media continued to grow throughout the 1970s. Railway workers and the public did not feel reassured by the claims made by SJ that their new herbicide Karmex 80 was harmless, and on two occasions, in 1984 and in 1986, SJ had to send for the police in order to clear the railways of protesters. Since then, sentiments have calmed down, but the largely negative opinion held by the Swedish people about herbicides, and herbicides used on railways in particular, have prevailed until this day.

3.3.3 Weed control on Swedish railways from 1974

When spraying operations were resumed in 1974, Karmex 80 was the primary choice for weed control on Swedish railways. Karmex had an overall good weed control effect on railways, although some *Galium* species became resistant after several years of usage (Torstensson *et al.*, 2002). Diuron, the active ingredient of Karmex, is currently proposed to be classified as a potential carcinogen ('Limited evidence of carcinogenic effect') by the European Food Safety Authority (EFSA, 2005)**,** but fortunately, no adverse health effects have been reported from the use of Karmex by Swedish railway workers. However, after a couple of years, effects on the environment immediately surrounding certain railway lines became evident. Pine trees (*Pinus silvestris*) up to 16 m from the track centre were killed and this could be attributed to the use of diuron. Investigations initiated by the Swedish Environmental Protection Agency revealed that roots from the dead pine trees had grown into the embankments and had taken up diuron. Furthermore, data suggested that diuron was very persistent and mobile in railway embankments (Torstensson, 1983; Torstensson, 1985; Torstensson *et al.*, 2002). These investigations served as a wake up call for the Environmental Protection Agency and for SJ, and showed for the first time that the fate of herbicides that were applied to railways could significantly differ from their fate in agricultural soils. It was decided that before herbicides could be approved for use on railways they needed to be tested in the railway environment. Consequently, a test programme was initiated in 1985, in cooperation with the Swedish University of Agricultural Sciences, with the purpose of finding herbicides that were both effective for weed control and acceptable from an environmental point of view (Torstensson, 2006).

In 1986, glyphosate came into use and it has been used continuously ever since. Glyphosate was initially sold under the name Roundup, but its manufacturer Monsanto did not want to risk the negative opinion people still held about

herbicide use on railways staining the name of their widely used agricultural product and so decided to sell glyphosate in an identical formulation but under a different name, Spectra, to the Railway Administration (Torstensson, 2006). Later, a new formulation called Roundup Bio came into use (Table 1). Glyphosate displays an overall good weed control effect when applied at a dose of 5 l/ha but has no effect on the railway problem weed horsetail (*Equisetum arvense*). However, although glyphosate is generally quite immobile in railway embankments, the findings of it, as well as its degradation product AMPA, in groundwater below the tracks indicate that an environmentally acceptable dose should not exceed 3 l ha^{-1} (Torstensson *et al.*, 2005). Such a low dose does not give acceptable weed control unless glyphosate is combined with another herbicide. Arsenal 250 (active ingredient imazapyr), which was used from 1994-2002, proved to be a suitable choice for such a combination. A mixture of glyphosate and imazapyr at a rate of 3+2 l ha^{-1} was shown to be both effective (even for horsetail) and environmentally acceptable (Torstensson & Börjesson, 2004). However, imazapyr was very mobile in railway embankments and it could be detected for extended periods in groundwater below the tracks if it had been applied in too high a dose (Börjesson *et al.*, 2004). Its use on railways was not accepted from 2002 by the Chemicals Inspectorate, a decision that was initiated by the owner of the preparation. Current research efforts (Figure 2) are aimed at finding new herbicides that can have both acceptable weed control and environmental behaviour, by themselves, or in combination with glyphosate.

Figure 2. Application of herbicides in a field experiment close to Falerum in Småland, summer 2005.

3.4 Alternative methods for weed control on railways

There are many restriction surfaces on Swedish railways such as for example rail yards and water protection zones, where the application of herbicides is not allowed, and these stretches of track need to be treated by alternative methods (Hansson *et al.*, 1995). Several non-chemical methods for weed control on railways have been tested by SJ, the Swedish Railway Administration and also by other railway operators that have similar needs (Eriksson *et al.*, 2004). Already in 1978 some experiments were conducted with the use of steam to control vegetation on Swedish railways (Skoog, 2006). Steam has also been tested in full scale trials for several years by the Canadian Pacific Railway, and in trials by the E&N Railway Company, in Austria and in Switzerland, but has not been adopted as a permanent technique (Anonymous, 2005a; Eriksson *et al.*, 2004). More recent research funded by the Swedish Railway Administration has concerned the use of hot-water (Hansson, 2002). In the US, at least one hi-rail for weed control of railway tracks with hot water has already been constructed and used successfully by the Asplundh company (Anonymous, 1994). However, hot water treatments share with other thermal measures the drawback that they only kill the above-ground parts of the plants. Therefore, in order to obtain an acceptable level of weed control the treatment needs to be repeated several times during one season. Hansson & Ascard (2002) estimated that 6 treatments per year would be required to achieve an effective level of control using this method. Furthermore, the speed at which the water treatment can be performed is relatively slow (0.9-8.3 km h^{-1}) and due to the large amounts of water that are required it may have a limited range (Anonymous, 1994). These limitations render it an impractical method for longer track sections. A train for weed control of railway tracks with infrared light has been developed and tested in Germany (Kreeb & Warnke, 1994). However, trials indicate that this technique has little potential for weed control on railways (Eriksson *et al.*, 2004). Flame weeding is generally considered to be more effective, have a higher operational speed and be cheaper than weeding with infrared light (Ascard, 1998), and in Germany, a full scale train for flame weeding has been developed and tested. However, the trials were cancelled because of problems with the equipment causing uncontrolled fires (Eriksson *et al.*, 2004). Freeze weeding with liquid nitrogen has been tested but is considered to have little potential for weed control because of low efficiency and high energy demands (Eriksson *et al.*, 2004). Application of hot biodegradable foam and UV-light are two recent techniques that have also been suggested for weed control on railways but for these further development is needed (Anonymous, 2005a; Eriksson *et al.*, 2004).

Preventive and constructional measures such as different vegetation barriers have been shown to be effective in limiting the need for chemical weed control where implemented (Eriksson *et al.*, 2004; Müller, 2001; Schroeder & Hansson, 2003) and there is a potential to use these methods in the construction of new railways. Ballast cleaning simultaneously removes weeds, but this is very slow, time-consuming and expensive (Müller, 2001). Other mechanical methods such as mowing and brush cutting are used to control weeds and brush in verges and slopes surrounding the railway (Huisman, 2001). However, mowing of the track

bed itself can be detrimental, since it leads to accumulation of organic matter and only intensifies management needs (Anonymous, 2005a). Manual weeding can be very effective in areas of manageable size (Anonymous, 2005a) but the work performance has been estimated to be only about 30 m^2 per hour and person (Müller, 2001) and the cost was estimated to be about 20 SEK m^{-2} in 1994 (Eriksson *et al.*, 2004). Hand weeding also has the drawback of being potentially risky for the personnel performing the work (Torstensson, 2001).

In conclusion, several of the alternative methods show potential to be used on rail yards and restriction surfaces where low operational speeds and limited range is not a problem, and where it is feasible to repeat the treatments several times during one growing season. However, for weed control of longer railway stretches herbicides still appear to be the most realistic option.

4 The railway track as a microbial habitat

The discovery in 1949 of nitrogen fixation in the photosynthetic bacterium *Rhodospirillum rubrum,* which was first seen in an experiment conducted on board a train travelling between St Louis and Madison, probably marks the starting point of the scientific field of railway microbiology (Gest, 1999). However, it was not until 1981 that the first report on the microbiology of some actual tracks was published. Smith and co-workers investigated the microbiology of some oil-contaminated urban tracks in Scotland and concluded that the track environment is 'arid, prone to great temperature fluctuations and is nutrient limited' (Smith *et al.*, 1981). They also saw a definite seasonal fluctuation in the numbers of microorganisms that could be isolated on agar plates, with the highest numbers being retrieved in the wetter winter and autumn months. In a more recent investigation of microbial properties of some metal-contaminated, long since closed, railyards, Murray *et al.* (2000) reported on some comparably high to very high substrate-induced respiration rates (SIR-rates), but according to the site descriptions these yards were so densely vegetated that they were probably more representative of meadows and forests than of railways. However, as can be deduced from Figure 3, the paper by Murray *et al.* (2000) hinted at an approximate SIR of 1.19 μg CO_2-C g^{-1} h^{-1} for railways that is still in use, and, in anticipation of section 5.2, this represents only a slight overestimation of the results obtained in **Papers I** and **II**.

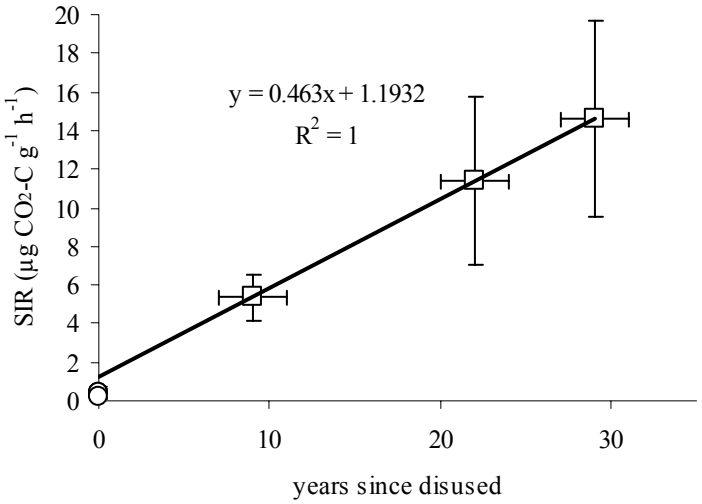

Figure 3. The relationship between the ageing of railway tracks and the size of their microbial biomass. Mean values of SIR rates ± standard deviations from railways still in use (○) come from **Papers I** and **II** and were not included in the regression. Mean values of SIR rates ± standard deviations from disused tracks (□; n=8 for each data point) were graphically obtained from Murray *et al.* (2000). The years that the railyards were disused are given in the original publication as in the 'late 60s', 'mid 70s' and 'late 80s', and since the field sampling was conducted in 1997 according to Ge *et al.* (2000), the SIR-values for the three railyards are plotted here as 29 ±2 years, 22 ±2 years and 9 ±2 years respectively.

Some limited measurements of the SIR of railway tracks have been carried out by Börjesson *et al.* (2004) using the same equipment as the author (**Papers I** and **II**). However, apart from that paper, the previously cited articles and the papers included in this thesis, no other reports on the microbiology of railway tracks have been published as far as this author is aware. There are, however, several surveys and much prior knowledge of the physical and chemical environment of the railway embankment which are of interest since they so to say set the stage for the microbes.

One obvious characteristic of the track bed and a major determinant of microbial life is its coarse texture (see section 2). As previously mentioned, this can give rise to arid and temperature-fluctuating conditions and furthermore, the coarser particles in a soil are generally considered to harbour smaller amounts of microbes than the finer ones (Certini *et al.*, 2004). On the other hand, substrate availability is increased in lighter soils and this may contribute to an enhanced microbial degradation of herbicides (Hassink *et al.*, 1994; Strange-Hansen *et al.*, 2004). Another factor that constrains microbial life is the limited amount of organic matter that can be expected to occur in railway embankments. As discussed in section 4.1, one of the major reasons why weeds are removed from railways is to limit the accumulation of organic matter in the track bed, and determinations of organic matter or organic carbon in railway ballast or subgrade regularly reveal that this struggle has been largely successful (Börjesson *et al.*, 2004; Torstensson

et al., 2005). Low amounts of organic matter are known to be synonymous with low microbial biomass and low microbial activities (Anderson & Domsch, 1989; Beyer *et al.*, 1999; Schnürer *et al.*, 1985) and these in turn are known to correspond to low rates of pesticide degradation (Anderson, 1984; Jones & Ananyeva, 2001; Torstensson & Stenström, 1986). Furthermore, it is known that low levels of organic matter in soil can decrease the functional diversity of the prevailing microbial communities (Degens *et al.*, 2000).

Railways can in many ways be likened to old industrial sites, in that they contain contaminants, many of which could potentially influence microbial life, from over a century of diverse activities. Organic pollutants include spills of oil and polycyclic aromatic hydrocarbons that come from the treatment of wooden sleepers with creosote (Carling *et al.*, 2000; Smith *et al.*, 1981; Wan, 1991). Common metal contaminants include arsenic, chromium, copper, lead and iron, which likewise may originate from wood preservatives, but also from the sharpening of the rails and from materials that were used in the construction of the track bed itself (Andersson, 2002; Carling *et al.*, 2000; Jansson, 2001; Sandström, 2003). Elevated levels of heavy metals in soil can decrease enzyme activities (Renella *et al.*, 2005), increase metabolic quotients (qCO_2) (Chander *et al.*, 2001) and hamper the microbial degradation of herbicides (Said & Lewis, 1991).

4.1 Methods used to study railway microbes

4.1.1 Sampling sites and sampling strategies

The two sampling sites for the studies of microbial biomass, activity and functional diversity (**Papers I** and **II**) were partly chosen on the grounds of being easily accessible by car and because they had a reasonably low traffic intensity. The characteristics of these two sites are described more thoroughly in **Paper I** but it should be noted here that the track between Mora and Älvdalen (referred to as the Mora track) only carries one train per day, going back and forth to a saw mill, and that it represents an older and less managed type of railway, whereas the track between Nässjö and Vetlanda (referred to as the Vetlanda track) is probably more representative of Swedish railways in general (Cederlund & Stenström, 2004). However, the choice of the Mora track as a study site can be defended, since it is probably not a bad representation of the type of weed infested tracks that is treated with herbicides and to which the results of this thesis could potentially be extended for predictive purposes.

In order to be able to properly quantify a microbial property and to identify its driving variables, it is important to try to characterize the inherent variability of the soil ecosystem studied (Parkin, 1993). This is even more important when studying a soil type that is previously uncharacterized with respect to the variability of the measured property, as is the case with the railway embankments investigated. Thus, the sampling strategy, more thoroughly described in **Paper II**, with samples taken at equidistances of 1, 10 and 100 m, was designed in order to get an idea of the scales of variation that would be relevant for the measured respirometric parameters. It was thought from the beginning that the two railways would represent two distinct levels of variability, since it was known from

previous investigations that the track bed in Mora was constructed from homogeneous sand, whereas the Vetlanda track bed had been constructed from a heterogeneous till. However, as it turned out, the till of the Vetlanda embankment had been overlayered by coarse sand and the texture was thus similarly homogeneous on both sites.

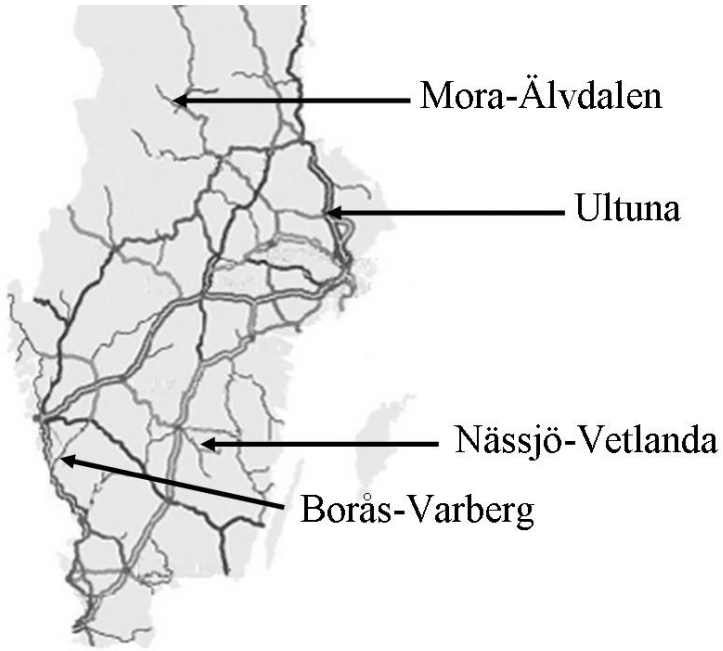

Figure 4. Overview of sampling sites for **Papers I-IV**.

For the study of the microbial degradation of diuron (**Paper III**) samples that were obtained for **Paper I**, and that were previously characterized with respect to SIR, were reused. For the study of MCPA mineralization and quantification of MCPA degraders (**Paper III),** samples were taken from one of the field sites used for testing herbicides for weed control, situated on the track between Borås and Varberg (referred to as the Varberg track). Soil was sampled from parcels that had received treatment for one or two subsequent years prior to sampling with a mixture of the formulations BASF MCPA 750 and Roundup Bio, as well as from previously untreated parcels, with the purpose of studying the microbial adaptation to the herbicide treatment. **Paper IV** is not a study of a railway embankment but of the Ultuna long-term fertilization experiment, which is situated in a field right outside of the Department of Microbiology. It is included in this thesis in order to provide an agricultural reference point against which the results from **Papers I-III** can be compared and contrasted. All the sampling areas are marked in Figure 4.

4.1.2 Microbial biomass and activity

4.1.2.1 Respiration

Soil respiration, the efflux of CO_2 from the soil, generally derives both from the metabolism that is needed to sustain and grow roots and associated mycorrhizae as well as from the degradation of organic compounds in soil by heterotrophic microorganisms (Ryan & Law, 2005). However, it was, primarily the latter group that was targeted in the respirometric assay used in present study, in which basal respiration and substrate-induced respiration (SIR) were measured under controlled and plant-free artificial conditions using an automated respirometer that continuously monitors the development of CO_2 from the soil (Nordgren, 1988). The basal respiration rate was used as a measure of general microbial activity in the soil and was determined when the respiration rate had reached a stable level after 6-8 days of incubation.

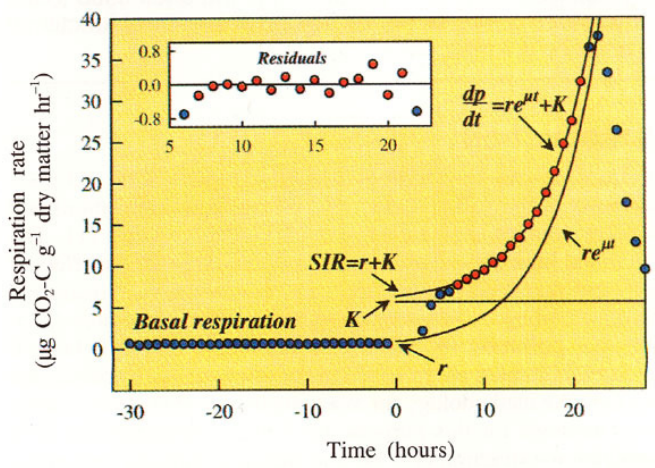

Figure 5. Kinetics of the respirometric measurement (from Stenström et al 1998).

4.1.2.2 Substrate-induced respiration (SIR)

After measurement of the basal respiration, a SIR substrate consisting of glucose at a saturating concentration and mineral salts carried by talcum powder was mixed into the soil samples and the resulting increase in the respiration rate was measured (Figure 5). Substrate-induced respiration was first proposed as a measure of soil microbial biomass by Anderson & Domsch (1978), and the basic idea is that the new respiration level, the maximum initial response, that is induced for a few hours by the addition of the substrate, is proportional to the size of the microbial biomass (Maartens, 1995). This in turn is based on the notion that the added substrate, usually glucose, can be utilized by all or at least by a proportionally sized fraction of the soil microorganisms. The high resolution of the respirometric assay (measurements every 30 min) allows for the kinetic derivation of the instantaneous rate of respiration upon glucose addition (Stenström *et al.*, 2001) and this is what was used as the SIR measurement in **Papers I-IV**.

4.1.2.3 Metabolic quotient (qCO_2)

The metabolic quotient (qCO_2) is calculated as the ratio of basal respiration rate to SIR and hence reflects the efficiency of the heterotrophic microorganisms to convert degraded organic matter into biomass. This measurement has been widely used as a stress indicator, and a high qCO_2 is thought to correspond to an elevated stress level and higher needs for maintenance energy (Anderson & Domsch, 1985; Chander *et al.*, 2001; Renella *et al.*, 2005). A more thorough discussion of its interpretation can be found in **Paper IV**.

4.1.3 Active microorganisms

4.1.3.1 *r* and *K*

The shape of the respirometric curve after the addition of the SIR substrate provides additional information about the composition of the microbial biomass since it reflects the size of the proportion of microorganisms that are actually using the glucose for exponential growth. This can be used to kinetically divide the SIR response into one growing fraction (*r*) and one fraction that only responds to the glucose addition by instantaneously increasing its respiration rate without subsequently increasing in numbers (*K*) (Stenström *et al.*, 1998; Stenström *et al.*, 2001). Small glucose additions prior to SIR measurement have been shown to induce a transformation of *K* into *r* (Stenström *et al.*, 2001) without leading to an increase in the total microbial biomass, and it has been postulated that the *r* and *K*-fractions of the microbial biomass, as they are derived in the respirometric assay, reflect two different physiological states, one active (*r*) and one dormant (*K*), and that soil microorganisms may alternate between these two states (Stenström *et al.*, 2001). It has also been observed by others that trace amounts of glucose added to soil may trigger the soil microbial biomass into activity (Nobili *et al.*, 2001), and it has been suggested that the size of the active fraction is governed by the presence of easily metabolizable organic substrates (Stenström *et al.*, 2001; Werf & Verstraete, 1987). Typically the *r*-fraction constitutes 5-20% of the SIR in agricultural soil without plants (Stenström *et al.*, 2001).

The use of the terms *r* and *K* can be somewhat confusing when used in the context of soil microbiology and it might be a good idea to elaborate a little on this terminology. The concept of *r* and *K* strategists or *r*-selected and *K*-selected organisms has been widely used in ecology, and a simplistic interpretation is that *r*-selected organisms are opportunistic organisms that favour high reproduction rates to parental care, and that are often found under disturbed or changing environmental conditions, whereas *K*-selected organisms are expected to be more prevalent under equilibrium conditions where the system is close to its carrying capacity (*K*), and where it is more advantageous to be competitive for the sparse resources that are available than to be fecund (Campbell, 1996). Thus, a typical *r*-selected organism would in this classical sense be a mouse, and a typical *K*-strategist would be an elephant or a human.

The terms *r* and *K*, as they are discussed in **Papers I-IV**, share some properties with this traditional concept, *e.g.* that the *r*-fraction of the microbial biomass is rapidly reproducing whereas the *K*-fraction is non-growing or growing only at a

very moderate rate. However, one important difference is that the *r* and *K* concept in soil microbiology is often used to discuss different phenotypes rather than different types of organisms. Thus, the *r*-fraction is thought to represent active, viable bacteria, whereas the *K*-fraction is thought to correspond to a dormant or viable but non-culturable (VBNC) state, potentially of the same bacterial species. It is generally considered that in response to starvation, bacteria can enter into a physiological state in which they retain at least a minimum of cell functions but loose their culturability on traditional agar media (Bakken, 1997; Kjelleberg *et al.*, 1993). The formation of such VBNC states and resuscitation of these into viable states have been demonstrated for many bacterial species (Ghezzi & Steck, 1999; Kaprelyants & Kell, 1993; Marsch *et al.*, 1998; Overbeek *et al.*, 1995; Wong *et al.*, 2004). These cells typically have an acquired starvation and stress tolerance and a smaller cell size than viable bacteria (Bakken & Olsen, 1987; Gasol *et al.*, 1995; Nayak *et al.*, 2005).

However, despite many efforts to elucidate the meaning of the terms, the definitions of 'viable', 'VBNC', 'active', 'dormant' and 'dead' microbial cells remain rather unclear (McDougald *et al.*, 1998). The idea that the formation of VBNC bacterial cells really represents an adaptive and reversible response to starvation and not just a gradual cellular deterioration has been questioned (Nyström, 2001). Furthermore, the 'activity', 'viability' or 'culturability' of a microbial cell is evidently very much dependent on how it is assayed (Davis *et al.*, 2005; Hahn *et al.*, 2004; Nielsen *et al.*, 2003; Zengler *et al.*, 2002). In order to try to shed at least some light on where the *r* and *K* concept of the respirometric assay fits into this somewhat obscure overall picture, an experiment was designed with the purpose of comparing the kinetically derived activity measure r with another widely used activity measure, CTC-staining.

4.1.3.2 CTC-staining

The staining of bacteria with 5-cyano-2,3-ditolyltetrazolium chloride (CTC) as a measure of respiratory activity has been widely used in environmental microbiology (Créach *et al.*, 2003; Sieracki *et al.*, 1999; Winding *et al.*, 1994). The principle of the assay is that CTC is transported into the cells where it acts as an artificial electron acceptor. Detection of the reduced form of the stain, the fluorescent CTC-formazan product (CTF), is thus indicative of an active electron transport chain in the stained cell (Rodriguez *et al.*, 1992). The method is generally considered to underestimate the number of active cells in soil compared to other measures of activity and to detect mainly the most active cells (Nielsen *et al.*, 2003). This could derive from the fact that the stain is toxic to microorganisms and that many bacterial cells may be killed before they have reduced enough CTC to allow them to be detected (Hatzinger *et al.*, 2003; Ullrich *et al.*, 1996).

4.1.3.3 Design of the comparative study

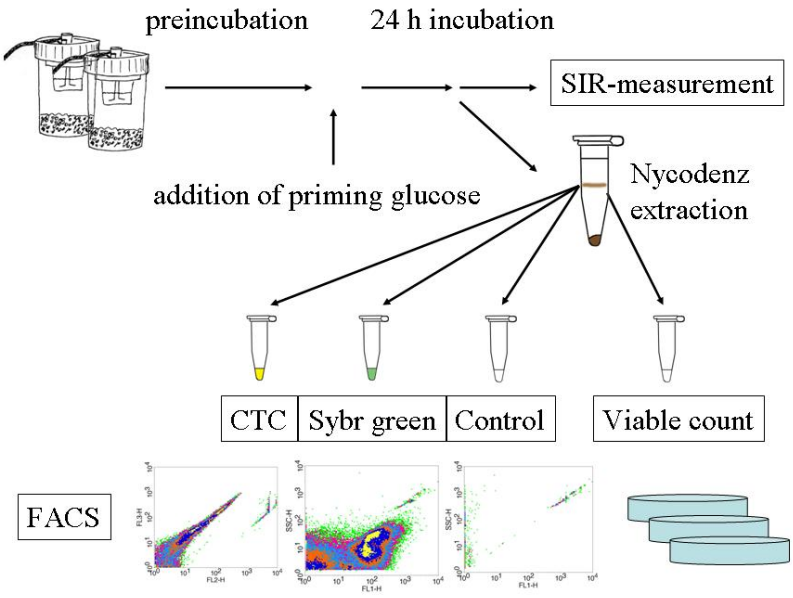

Figure 6. Experimental design of the comparative study

Soil was sampled from the agricultural soil Ulleråker, sieved (<4 mm) and stored in the dark at 4 ºC. Soil samples (20 g) were weighed into respirometric jars and were incubated in the respirometer at a constant temperature of 22 ºC until a stable basal respiration rate was reached. Then, priming amounts of glucose (0-2.20 mg g^{-1} dry wt soil), were mixed into the soil samples, with talcum powder as a carrier. The lowest glucose concentrations were intended to partially activate the microbial biomass without inducing any growth, whereas the highest concentrations were intended to completely transform the biomass from its *K* to its *r* state and simultaneously induce detectable growth as described by Stenström *et al.* (2001). Twenty-four hours after the addition of the priming glucose concentrations, half the incubated jars were removed from the respirometer. To the remaining jars, a SIR substrate was added and the parameters *r* and *K* were kinetically derived as described in section 4.1.3.1. From the removed jars, soil samples (1 g) were taken and bacteria were extracted from these by a Nycodenz density gradient extraction (Bakken & Lindahl, 1995; Lindahl & Bakken, 1995) using a protocol modified from (Maraha *et al.*, 2004). The extracted bacterial cells were split into four fractions: one stained with CTC (4.5 mM), one stained with the nucleic acid stain SYBR green II (for estimation of the total number of cells), one unstained control and one fraction that was subsequently diluted and used for viable counts on R2A agar. The stained and unstained control fractions were analysed using flow cytometry together with a known concentration of green fluorescent microspheres as an internal standard (Maraha *et al.*, 2004; Tombolino *et al.*, 1997). An overview of the experimental setup is shown in Figure 6. The flow cytometer can rapidly

count large numbers of microbial cells and sort them based on how they scatter light; forward scatter (FSC) and side scatter (SSC), and based on their fluorescence (Veal *et al.*, 2000) and this was used to quantify different fractions of the extracted cells based on their size (which corresponds to FSC and SSC) and based on their respiratory activity (CTC+/- cells) (Figure 7). Factors tested were the effects of different priming glucose concentrations, different staining times and the influence of glucose additions while staining the cells.

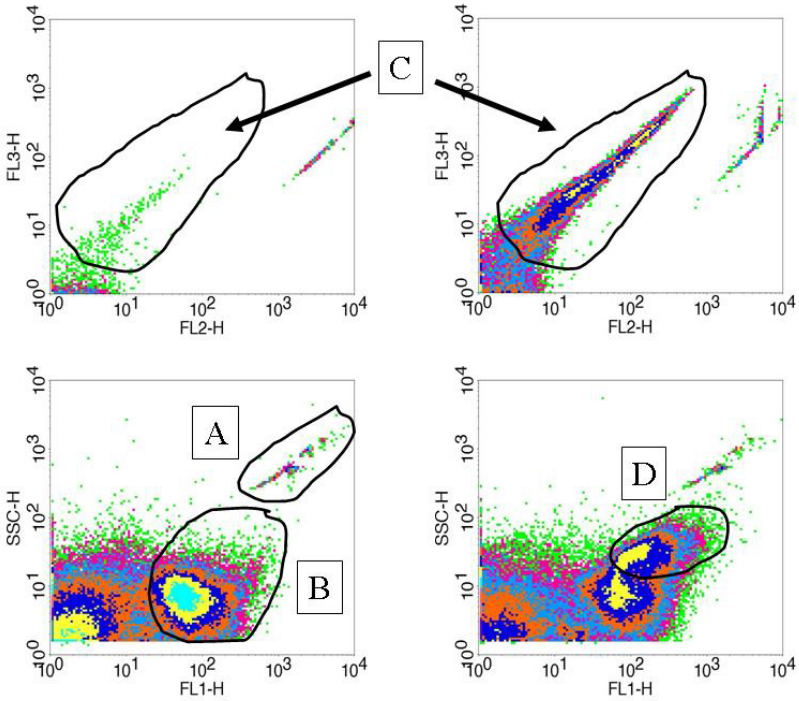

Figure 7. Detection of different fractions of microbial cells using the flow cytometer: Comparison between extract from control soil (left) and glucose-amended soil (right): A = internal standard; B = SYBR green stained cells; C = CTC+ cells; D = subpopulation of large SYBR green stained cells.

4.1.3.4 Results from the comparative study

There was a linear relationship between the increase in the parameter *r* and the low amounts of glucose that were added to the soil, and this is in good agreement with what was observed by Stenström *et al.* (2001). However, the linearity did not extend to higher levels of glucose amendments, where the amount of *r* that was induced per unit of glucose appeared to level off (Figure 8).

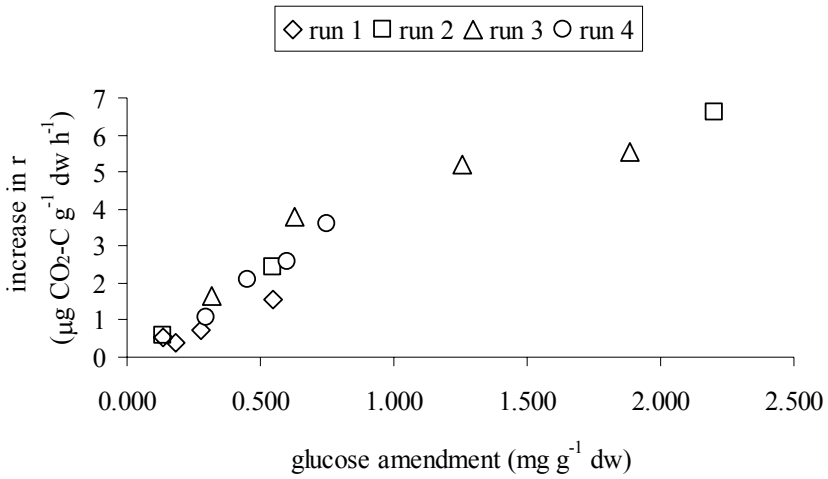

Figure 8. Increase in the parameter *r* as a function of the amended amount of glucose. Data from four different runs on the respirometer.

The parameter *r* was subdivided into three fractions: *initial-r,* representing the amount of active microorganisms in the unamended control soil; *growth-r,* representing the active microorganisms that can be explained by a corresponding increase in the SIR, *i.e.* the cells that have been formed from growth on the added glucose; and thirdly *transformed-r,* representing the fraction of *r* that has been formed by a transformation from *K* (Figure 9). When this was done it became evident that microbial growth was induced already at very low glucose concentrations. This is not consistent with the findings of Stenström *et al.* (2001), who reported that growth was only induced by glucose additions that were high enough to completely transform the microbial biomass into its *r*-state. One probable explanation for this apparent discrepancy is that in the study of Stenström *et al.,* the priming glucose additions were mixed into the soil 4 days prior to the SIR-measurement whereas in the present study they were administered only 24 hours prior to its assessment. A longer time period between priming and measurement in the Stenström *et al.* study could have allowed cell numbers to recede to their background value.

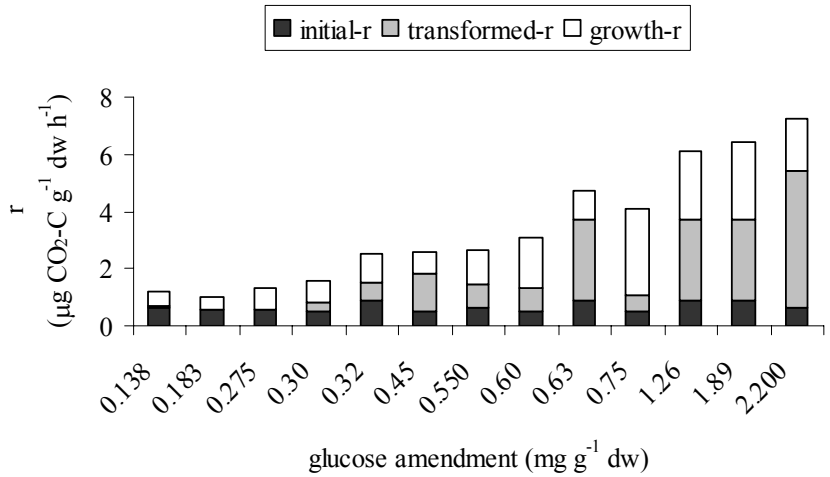

Figure 9. The parameter *r* as a function of glucose amendment split up into three subgroups: initial-r = the fraction of *r* that is present in the soil without glucose amendment; growth-r = the fraction of *r* that corresponds to a simultaneous increase in SIR; and transformed-r = the fraction of *r* that is formed by transformation of *K* to *r*.

The number of cells that were stained by CTC (CTC+ cells) also increased linearly with the amount of added glucose, and *r* correlated to CTC+ ($r^2 = 0.81$). However, the proportion of active cells, calculated as the ratio of CTC+ cells to SYBR green stained cells, did not display a linear relationship to the proportion of active microbial biomass, calculated as the ratio of *r* to SIR (Figure 10).

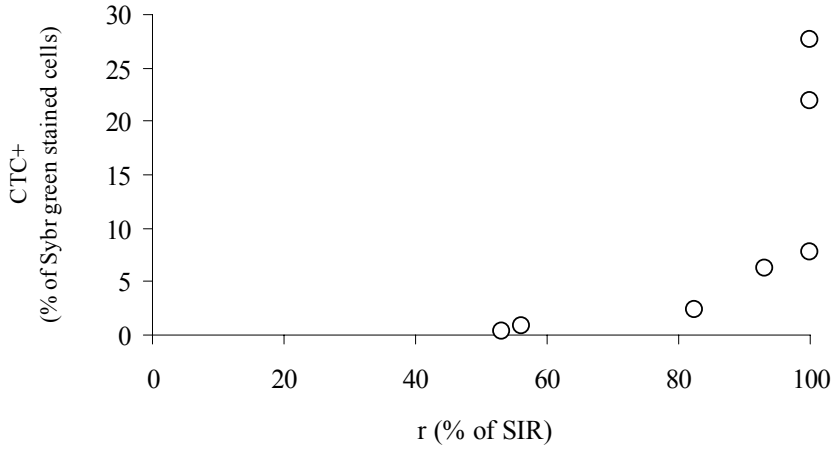

Figure 10. The percentage of active cells as assayed by the CTC-staining technique versus the percentage of active cells assayed by the respirometric approach.

It appeared that the percentage of *r* was a much more sensitive measure of the effects of the glucose amendments than the percentage of CTC+ cells. While the SIR could be completely transformed into its *r* state, the CTC+ cells never constituted more than 30% of the total number of cells. Since SIR is determined under glucose saturated conditions, it was hypothesized that the presence of a substrate while staining the extracted cells would lead to a better detection of respiratory activity by CTC. It was observed that the addition of glucose in the staining procedure increased the numbers of CTC+ cells without increasing the number of SYBR green stained cells, and this is consistent with what has been reported by others (Yoshida & Hiraishi, 2004). However, even this improvement of the staining procedure did not yield comparable estimates of the active fraction by the two methods. Prolonged staining times (3→9 h) did not affect the staining efficiency at all.

A source of discrepancy between the two methods could be that while the flow cytometry by necessity only targets extracted (mainly bacterial) cells, the SIR is measured directly in the soil samples. It is possible that active microorganisms may be disproportionately less extracted by the Nycodenz method, and even if a representative fraction is extracted, *in vivo* activity does not necessarily translate into activity *in vitro*.

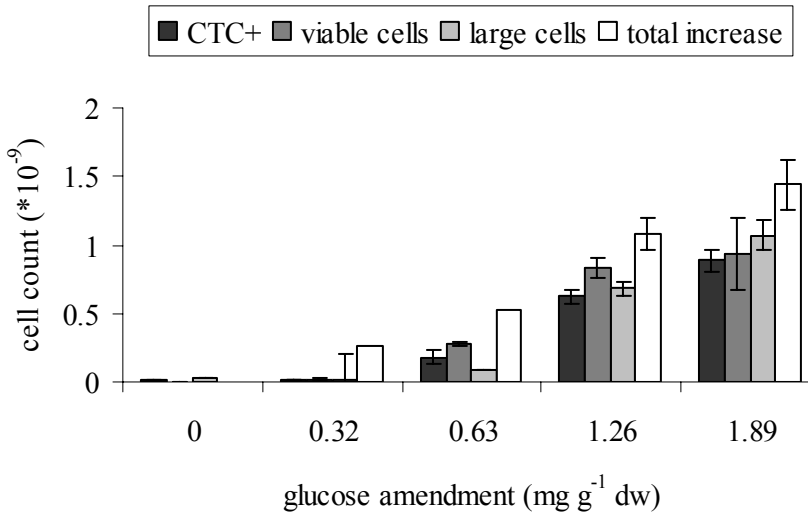

Figure 11. Number of CTC stained cells, viable cells assayed as colony forming units on R2A, large cells detected as a subpopulation of the SYBR green stained cells separated by FSC and SSC on the flow cytometer and increase in the total number of SYBR green stained cells as a function of glucose amendments.

The numbers of CTC+ cells were of almost exactly the same magnitude as the numbers of viable cells estimated by plate counts on R2A agar media. This is consistent with the findings of Créach *et al.* (2003), who concluded that CTC was a better estimator of cell viability than cell activity. Furthermore, the numbers of CTC+ cells were also comparable to the numbers of large cells gated by the flow

cytometer. The glucose induced increases in SYBR green stained cell numbers were consistently somewhat higher than for the other measures (Figure 11).

Thus, it appears that the priming glucose amendments induced growth among the soil bacteria, and that it was mainly a population of large, CTC-active and viable cells that was formed. No transformation of dormant cells into active ones could be observed using the CTC staining assay.

The SIR estimates were consistently well correlated with the number of SYBR green stained cells (r^2 = 0.94-0.97) and this seems to suggest that the SIR measure as such is a credible biomass measure. Concerning the parameter *r*, it is certainly a good indicator of the availability of easily degradable substrates in the soil. However, it is possible that the presence of a population of glucose-responsive growth-compatible cells may obscure the respiratory responses of less active or less glucose responsive cells in the soil. Thus, the parameter *r* may very well overestimate the proportion of active microbial cells just as the CTC staining procedure probably underestimates it.

4.1.4 Functional diversity

The functional diversity was characterized in **Paper II** by using Biolog ECO plates that contain a set of 3x31 presumably ecologically relevant carbon sources (Insam, 1997). The ECO plate provides similar discriminatory power for heterotrophic communities as the more widely used Biolog GN plate and has been recommended for environmental samples (Choi & Dobbs, 1999; Classen *et al.*, 2003). Biolog plates were first used for characterization of the functional versatility of heterotrophic microbial communities in soil, *i.e.* determination of their so-called community-level physiological profiles (CLPP), by Garland and Mills (1991). The principle of the assay is that diluted environmental samples, or microorganisms extracted from environmental samples, are inoculated into the wells of a Biolog microplate. There are about as many ways to extract and inoculate the plates as there are different studies, but for soil it is considered most sensible to inoculate the plates with a soil suspension rather than to try to remove soil particles by filtration or centrifugation prior to inoculation (Balser *et al.*, 2002; Preston-Mafham *et al.*, 2002). Each well of the plate contains a carbon source, some nutrients and the dye tetrazolium violet, which acts as an electron acceptor and forms a violet colour when it is reduced, and microbial utilisation of the carbon sources can thus be detected at a wavelength of about 590 nm with, for example, a plate reader (Preston-Mafham *et al.*, 2002).

Advantages of the CLPP method are that it is relatively cheap and easy to use and that it (potentially) provides both qualitative and quantitative information on the heterotrophic functions of the studied soil (Garland, 1997; Nannipieri *et al.*, 2003). Disadvantages include that it is a culture based approach and that soil fungi are unable to reduce the tetrazolium dye (Classen *et al.*, 2003; Konopka *et al.*, 1998). Furthermore, the response of the plate is highly dependent on the size of the initial inoculum, and failure to standardize the inoculum size may necessitate some kind of scaling or normalization procedure (Garland, 1996; Garland, 1997). Such

scaling may distort the substrate utilization pattern of the plates if the differences between the initial sizes of the inocula were too large (Garland *et al.*, 2001; Howard, 1997). For further elaboration on this theme the reader is referred to **Paper II**. The measures that have been obtained from the plates or that have been calculated from the plate readings, such as single point readings, average well-colour developments (AWCD), trapezoid areas, different kinetic parameters, diversity indices *etc.*, are many (Garland, 1997; Garland *et al.*, 2001; Guckert *et al.*, 1996; Harch *et al.*, 1997) and it is not always clear what they represent. In **Paper II**, the multivariate analysis of the plate responses was performed for plates with comparable AWCD but different incubation times as outlined by Garland (1997), and substrate richness, *i.e.* simply the number of positive well responses, was used as a straight-forward measure of functional diversity. Diversity also characterizes the statistical approaches that have been used to deal with the data from Biolog plates, such as different ordination methods, discriminant analysis, cluster analysis, self-organizing maps, multivariate analysis of variation *etc.* (Hacket & Griffiths, 1997; Hitzl *et al.*, 1997; Leflaive *et al.*, 2005; Palojärvi *et al.*, 1997). In **Paper II** another approach, based on generalized linear models, is introduced. It has the advantage that the dependence on explaining soil variables such as pH or organic matter content can be inferred even for discretely distributed variables such as substrate richness. For more details on this procedure the reader is referred to the Materials and Methods section of **Paper II**.

4.1.5 Structural diversity

It is known that only a minor fraction of the microorganisms in soil are cultivable using traditional culturing techniques (Torsvik *et al.*, 1990; Torsvik *et al.*, 2002), and while a CLPP may provide useful information on the functions that are carried out by microorganisms in a soil, it is uncertain how well it reflects the microbial diversity. In order to avoid the biases introduced by culture based methods structural approaches that rely on the extraction and characterization of cell constituents such as phospholipid fatty acids (PLFA) and DNA are increasingly utilized (Hill *et al.*, 2000). These methods are generally more sensitive for the detection of community changes than functional approaches, while on the other hand the inherent functional redundancy of soil microbial communities renders it difficult to link diversity or community structure assayed in this way to any function (Enwall *et al.*, 2005; Griffiths *et al.*, 2000; Griffiths *et al.*, 2001; Nannipieri *et al.*, 2003). **Paper IV** represents an attempt to link potential ammonia oxidation (PAO), which is carried out by a relatively defined group of bacteria, to the community structure of these ammonia oxidizing bacteria (AOB). The community structure of the AOB was assayed with denaturing gradient gel electrophoresis (DGGE), and the total bacterial community structure of the soil was estimated with terminal restriction fragment length polymorphism (TRFLP). These two PCR-based fingerprinting techniques allow for the separation of PCR-fragments of different sequences in the case of DGGE (Muyzer *et al.*, 1993), and of restriction fragments of different size in the case of TRFLP (Marsh, 1999), and can be used to target either total bacterial communities or subgroups of bacteria depending on the choice of primers for the PCR reaction (Head *et al.*, 1998).

4.1.6 Microbial degradation of herbicides

Paper III is a study of the microbial degradation of the herbicides diuron and MCPA in fine material of railway ballast. The herbicides were chosen on the basis of having different degradation kinetics, diuron being generally cometabolically degraded (Hill *et al.*, 1955) and MCPA metabolically degraded (Audus, 1951). The purpose was to illustrate the principal differences between these two types of environmental behaviours in a railway setting. Diuron is no longer used on railways in Sweden (see section 4.2.3) but is still widely used *e.g.* in the USA (Mengistu *et al.*, 2005). One reason for its popularity is its long residual activity (Madin & Rutherford, 1984), a quality that has also led to it being a groundwater contaminant of concern (Skark *et al.*, 2004). MCPA is also widely used on railways and is currently being investigated as an alternative for the Swedish railways. It is hoped that the metabolic degradation kinetics will render its use less problematic from an environmental perspective.

Diuron degradation was measured on HPLC which gives the advantage that the formation of the ecologically relevant metabolites DCPMU, DCPU and DCA also can be monitored (Figure 12). These metabolites have been shown to have higher non-target toxicity than the parent compound (Tixier *et al.*, 2001) and the importance of evaluating their dissipation has been emphasized (Giacomazzi & Cochet, 2004). The disadvantages of the setup are that the number of measurements is limited since destructive sampling is a necessity, and that no information on the mineralization is obtained.

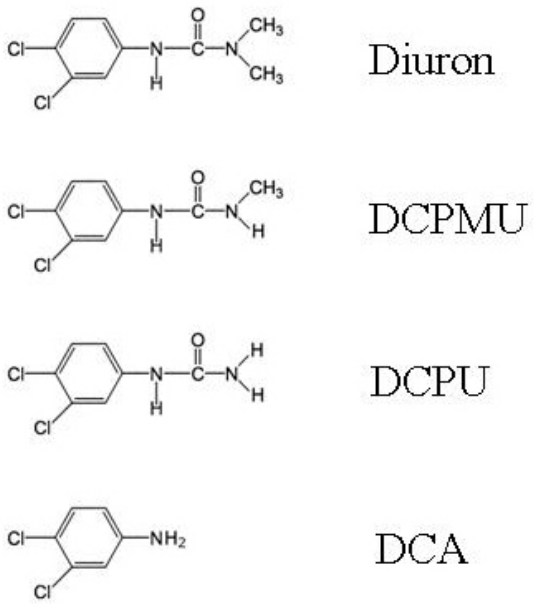

Figure 12. Diuron and its degradation metabolites whose formation was monitored in **Paper III**. Diuron (-(3,4-dichlorophenyl)-1,1-dimethyl urea); DCPMU (1-(3,4-dichlorophenyl)-3-methyl urea); DCPU (1-(3,4-dichlorophenyl) urea); DCA (3,4-dichloroaniline).

Mineralization of radioactively labelled MCPA was monitored by measuring the release of ^{14}C-CO_2. This approach makes it possible to obtain kinetic information of relatively high resolution since the sampling is non-destructive, but on the other hand it provides no information on degradation products. Kinetic parameters of interest include the initial activity (qN_0) and specific growth rate (μ) of the MCPA degraders, which in **Paper III** are compared and discussed in relation to the number of MCPA degraders in the soil as determined by a ^{14}C-MPN-approach (Lehmicke *et al.*, 1979). A simultaneous study on mineralization (release of ^{14}C-MCPA) and degradation of MCPA (measured on GC) allowed for the calculation of yield estimates (Y) in the field samples, *i.e.* the numbers of microbial cells formed per unit of MCPA degraded. For further details the reader is referred to the Materials and Methods section of **Paper III**.

4.2 Microbial life in the track bed

4.2.1 Microbial biomass and microbial activity

Figure 13 gives an illustrative overview of the SIR rates that were determined in **Papers I-IV** and clearly demonstrates the overall insignificant size of the microbial biomass on railways. Here it should be emphasized that all the microbial analyses were determined on the sieved fine material of the railway ballast and that it was not feasible to estimate the stoniness of the sampled track beds, which in some instances was considerable. Hence, it is likely that all the respiration rates that are reported in this thesis are actually overestimations of the respiration rates, expressed as released CO_2-C g^{-1} ballast.

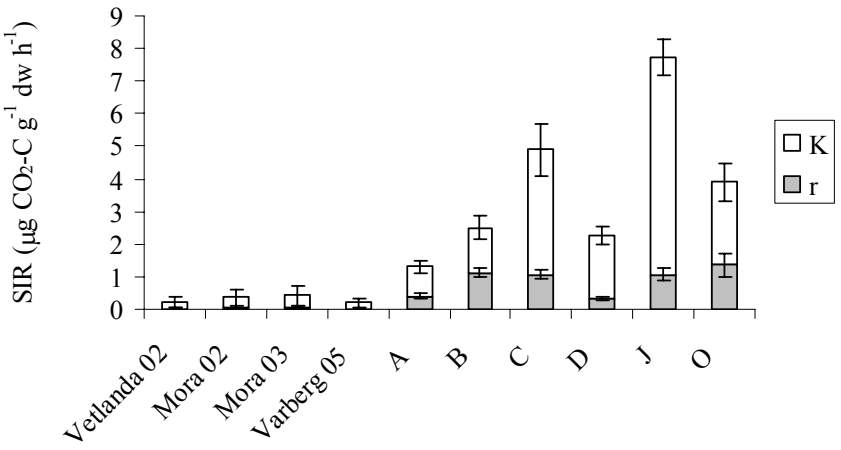

Figure 13. Comparison of the size of the substrate-induced respiration (SIR) on railways and in differently treated agricultural soils plotted as the sum of the parameters r ± standard deviation and K ± standard deviation. Data for Vetlanda 02 (n=47) and Mora 02 (n=61) from **Paper 1**, data for Mora 03 (n=20) from **Paper II**, data for Varberg 05 (n=12) from **Paper III** and data from the Ultuna long-term fertilization experiment (A-O; n=3 in each case) from **Paper IV**. Treatments were: A = unfertilized treatment without crop; B = unfertilized with crop; C = calcium nitrate $Ca(NO_3)_2$; D = ammonium nitrate $(NH_4)_2SO_4$; J = solid cattle manure; O = sewage sludge.

As can be deduced from Figure 13, the railway SIR rates were most similar to the SIR rate from the non-cropped treatment A of the Ultuna long term fertilization experiment, which has received no fertilizers and no organic inputs for almost 50 years, whereas the rates were several-fold lower than those determined in soil from cropped plots that have received more traditional fertilizer treatments. This illustrates the importance of organic matter (in this case inputs through fertilization, root exudates and decaying crop parts) in determining the size of the microbial biomass and, as outlined in **Paper II**, loss on ignition, *i.e.* the organic matter content, was indeed the most important determinant of SIR in the railway embankments. This is of course very logical: the microorganisms are present where there is something to 'eat', and where 'food' is scarce microbial numbers are low.

The dearth of resources led to characteristic positively skewed distributions of the measured microbial properties. Overall low amounts and activities were interrupted by occasional hot spots of activity where some leaves or the remains of a weed were decaying in the track bed (Figure 14). Spatial variability was high and no covariance structures were revealed in the dataset indicating that autocorrelation was sustained on a shorter than 1 m scale.

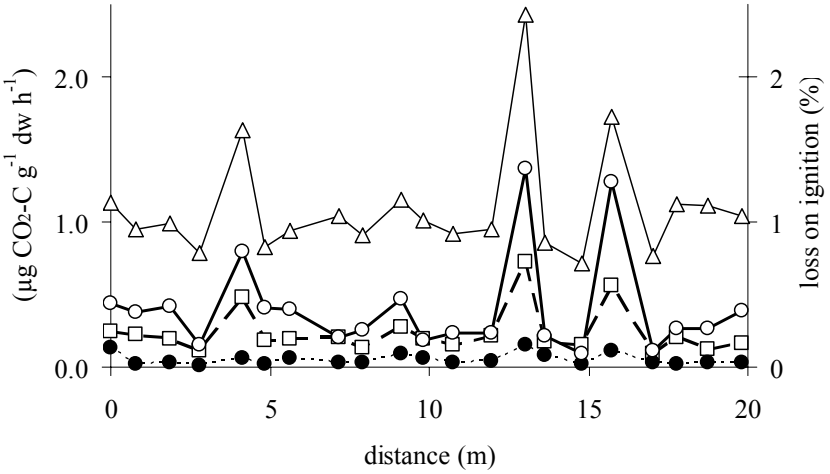

Figure 14. Spatial variability in loss on ignition (Δ), SIR (○), basal respiration (□) and r (●) determined in topsoil sampled at the centre of the track in Mora 2003.

Organic matter contents and SIR rates generally appeared to be higher at the sides of the railway embankment than in the centre of the track and this is probably an effect of higher litter fall from the surroundings as well as a higher incidence of invading weeds on the sides of the embankment. Basal respiration and to some extent r behaved similarly to SIR in most instances. Organic matter was an equally critical factor; however, for these measures of activity the water content appeared to be an even more important covariate. Both basal respiration and the active fraction r were higher when sampled during moister conditions in the autumn than

when sampled in the summer, and this is in good agreement with the observations of Smith et al. (1981). The fraction of the SIR that was active was typically below 5% in the summer, but increased to a median value of 11.6% in the autumn, which is a value more comparable to that of an uncropped agricultural soil. It may very well be that microbial activity is restrained by low water potentials during large parts of the year.

The metabolic quotients (qCO_2) of the railways samples did not correlate to pH as in the case of the soil from the Ultuna long-term experiment (see **Paper IV**). The mean railway qCO_2 values were comparable to the highest values obtained from the Ultuna soil. However, because of the higher basal respiration rates in Mora in the moister autumn samples, the qCO_2 was almost doubled for that sampling occasion. High qCO_2 values have mostly been interpreted as indications of elevated stress levels; however, the quotient does not necessarily have such a straightforward interpretation. Just as conditions that hamper the formation of microbial biomass, conditions favourable to respiratory activity also yield higher quotients, and this would appear to be the case in the railway samples that were obtained in the autumn.

4.2.2 Functional diversity

Functional diversity, measured as substrate richness, was clearly lower in the railway in Mora than in the agricultural soils to which it was compared. A median value of 19 (out of 31) carbon sources were utilized in railway topsoil samples and a median of 10.5 carbon sources in the subsoil (Figure 15).

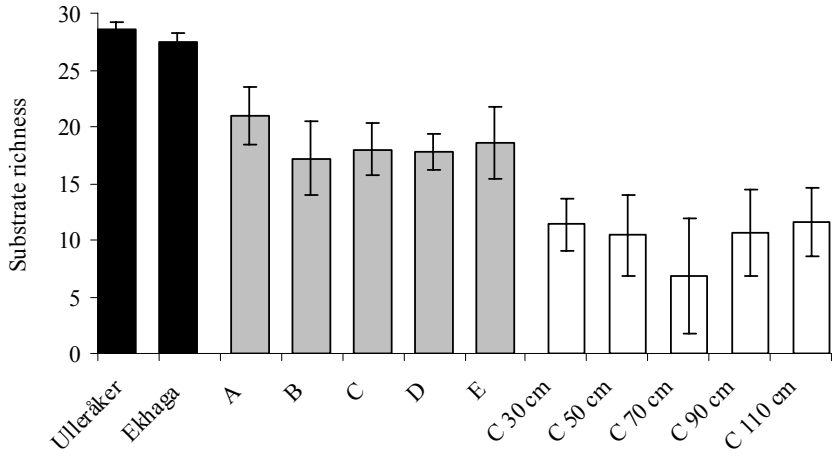

Figure 15. Mean values ± standard deviation of substrate richness, *i.e.* the number of carbon sources (0-31) that were utilized on Biolog ECO plates assayed after ten days of incubation. Comparison between two agricultural soils (Ulleråker and Ekhaga), five topsoil profiles (A-E) and five subsoil profiles (C 30-C110) from the railway embankment in Mora.

Within this overall pattern, variability was considerable and the number of carbon sources that were utilized on the plates ranged from 12-24 CS in the topsoil and from 2-17 CS in the subsoil. Substrate richness co-varied with SIR and thus it

appeared as though many functional groups of microorganisms were rare and that some of these were lost where the biomass is low. The multivariate analysis of the pattern of relative utilization of carbon sources suggested that it was not always the same functional guilds that were lacking in the heterotrophically less diverse samples. This would indicate that functional groups of microorganisms are largely randomly distributed on the railway tracks.

4.2.3 Microbial degradation of herbicides

Cometabolic microbial degradation of diuron in the railway ballast followed first order kinetics and thus did not support growth of the degrading microorganisms. The half-lives determined in **Paper III** ranged between 122-365 days and correlated to loss on ignition and SIR, and considering the high variability in SIR, it may well be equally variable in the field. Both of the demethylated metabolites DCPMU and DCPU were detected in all incubated samples and appeared to accumulate in most of them. High levels of DCPMU in particular is consistent with previous observations in railways after treatment with diuron (Torstensson, 1983; Torstensson, 1985). When these metabolites are taken into account, the half life estimates for diuron are almost doubled.

MCPA mineralization is growth-linked and relatively rapid as compared to diuron degradation. Mineralization in the railway ballast was clearly enhanced in soil from sections of the railway that had received previous treatments with MCPA. The time required for 50% mineralization of MCPA was decreased from 45.9±8.2 days to 13.8±11.3 days in these soils. This enhancement appears to derive from the fact that a number of MCPA degraders prevail in the railway from the previous year's application of MCPA. Yield estimates were low compared to estimations from liquid cultures and were correlated to the nitrogen content of the ballast, indicating that the formation of microbial cells from MCPA is limited by nutrient availability on the railways. Considering this and the high variability in degradation times that was seen in the previously treated samples, it cannot be ascertained that degradation will be enhanced everywhere on the track following an application of MCPA.

5. Informed use of herbicides on railway tracks

In the previous section, an image of the railway track bed as a relatively sparsely populated, highly variable, functionally non-redundant and nutrient limited microbial ecosystem was sketched. In this concluding section, some applicable strategies for the use of herbicides on railways that take this microbial paucity into account are outlined.

Several studies have established that the dose is an important determinant of the likelihood of an herbicide applied to a railway track subsequently leaching to groundwater (Börjesson *et al.*, 2004; Torstensson *et al.*, 2005). Thus, the use of

low-dose herbicides, or herbicides used at reduced doses, would be appropriate from an environmental point of view.

However, the accumulated risk for groundwater contamination over time depends not only on the concentration of the herbicide but also on how fast it is degraded in the ballast. A suitable concept for risk assessment of herbicides that integrates both the dose at which they are applied and their dissipation rate might be their 'availance', described by Hartley & Graham-Bryce (1980). This measure can be calculated as the integral of the concentration with respect to time, *i.e.* the area under the concentration curves in Figure 16. For typical first-order degradation kinetics, the decline in concentration over time can be described by:

$$c = c_0 e^{-kt},$$

where c is the concentration at time t, c_0 is the initial concentration and k is the first-order rate constant. The total integral of this equation is:

$$\int_0^\infty c\,dt = -\frac{c_o}{k} e^{-kt}\Big|_0^\infty - 0 + \frac{c_0}{k} = \frac{c_0}{k} = \text{availance}$$

This illustrates how even low dose herbicides (*i.e.* low c_0) may pose a significant risk to the groundwater if they have a slow degradation rate (*i.e.* low k).

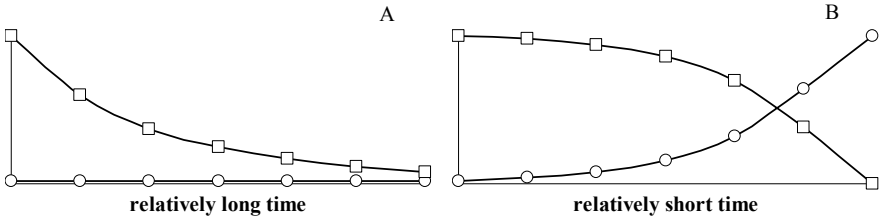

Figure 16. Principal differences between first-order (A) and growth-linked (B) degradation kinetics of herbicides. Cometabolically degraded herbicides degrade at a rate that is proportional to the remaining concentration (□) and thus the degradation rate decreases with time, whereas cell numbers (○) remain at a constant level. Metabolically degraded herbicides induce growth among their degraders and hence cell numbers and degradation rate increase exponentially.

Considering the overall low microbial amounts and activities on railway tracks, it is not surprising that cometabolically degraded herbicides, such as exemplified by diuron, are generally very slowly degraded. Thus, choice of herbicides that support growth-linked degradation kinetics, *e.g.* MCPA, would be much more sensible from an environmental point of view. These herbicides tend to be degraded faster, and the degradation rates would be further enhanced if the herbicide were for several years on the same track.

The geographical distribution of microbial biomass and activity in the track bed appears to be largely linked to inputs of organic matter from falling litter and plant

growth in the track bed, such that it might even be appropriate to refer to it as a ‘weed-ographic’ distribution. This discovery would very much seem to favour the idea of using ‘weed-seeker’ or ‘weed-eye’ techniques where the herbicide is applied selectively to where weeds are present on the track. Such approaches would not only reduce the dose applied considerably (Antuniassi *et al.*, 2004) but would also directly target the most microbiologically active parts of the railway track, thus increasing the probability of fast degradation.

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